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| CLEVELAND, O. | |
| Western Reserve University | October 19, 1959 |
| MINNESOTA | |
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## Inhibition of Foreign Spleen Reaction by Inactivation of Donor Cells with Recipient Antigen. (25305)

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Homologous lymphoid cells from adult mice implanted into neonatal(1) or irradiated adult (2,3) recipients cause a lethal reaction that has been ascribed to production of antibodies by the grafted cells against the tissues of the host. Since, however, the probability of death varies markedly with the number and immunologic reactivity of the cells inoculated, the recipient's age, and the dose of radiation (2), factors other than the incompatibility of donor and recipient influence the ability of the implanted cells to react lethally. We therefore postulated that the implanted cells are unable adequately to react against the recipient if exposed to too high a concentration of antigen and tested the hypothesis by heavily exposing the donor cells *in vitro* to recipient antigen prior to their implantation. Results of these experiments form the basis of this report.

*Methods.* Twelve-week-old female (C57BL $\times$ 101)$F_1$ mice were exposed to 500-600 r whole-body X radiation under the following conditions: 250 kvp, 30 ma, 3 mm of Al added filtration, 93.7 cm target-to-mouse distance, hvl 0.44 mm of Cu, 85 r per minute. Within one hour after irradiation, each mouse was injected intraperitoneally with a suspension of C57BL spleen cells in Tyrode's solution, receiving the equivalent of one donor spleen ($\sim 150 \times 10^6$ nucleated cells) in 0.5-2.0 ml. The spleen cell suspensions were prepared in a Potter-Elvehjem glass homogenizer from pooled intact spleens of young adult C57BL male donors. In some instances (Table I), the spleen cell suspensions were previously incubated with donor or recipient liver, as follows: to the suspended spleen cells was added a suspension of liver cells from young adult mice, prepared with a Potter-Elvehjem glass homogenizer, one spleen being added to one liver in the first, and six spleens to one liver in subsequent experiments. Benzathine penicillin G was also added, 300-600 units per ml.

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TABLE I. Effects of Pretreatment of Implanted C57BL Spleen Cells* on Survival of (C57BL × 101)$F_1$ Recipients.

| | | Recipients | | |
|---|---|---|---|---|
| Material inj. | X-ray dose (r) | Fraction dying in 35 days | Fraction with donor-type RBC | Mean time of death (days) |
| 1 C57BL spleen | 600 | 32/32 | | 19 |
| + 1 C57BL liver | 500 | 5/5 | | 16 |
| + 1/6 ” ” | 600 | 32/32 | | 19 |
| + 1 (C57BL × 101)$F_1$ liver | 500 | 2/5 | 3/3 | 26 |
| + 1/6 ” ” | 600 | 3/24 | 11/21 | 19 |
| None | 600 | 1/32 | | 7 |

* For description of spleen cell suspensions see text.

The pooled liver-spleen suspensions were refrigerated at ~10°C for about 19 hours before injection. The suspension was mixed by gentle agitation; one-spleen-dose aliquots were withdrawn for each recipient animal and injected through a 22-gauge hypodermic needle into the peritoneal cavity. After inoculation, the recipients were observed until death or for periods up to 200 days. The animals were caged in groups of 5-10 and had free access to food and water at all times. At intervals after inoculation, the erythrocytes of randomly selected mice were tested by starch gel electrophoresis(4) and hemoglobin solubility characteristics(5) to determine whether they were of donor or recipient type.

*Results.* All recipients of spleen cells plus donor liver homogenate or spleen cells alone were dead within 35 days, whereas most recipients of spleen cells plus recipient liver homogenate survived indefinitely (Table I). The few deaths in the latter group occurred slightly later than those in the former groups. Pathologic changes in the mice that died resembled those described earlier(2,6). In the majority of survivors, erythrocytes of donor type appeared in the circulating blood in increasing numbers after the 15th day, indicating transplantation and proliferation of donor-type erythrocyte progenitors. Irradiation (600 r) alone killed only one of 32 mice exposed.

*Discussion.* Inhibition of the foreign spleen reaction by incubation of the grafted cells *in vitro* with recipient-type antigen cannot be ascribed to killing of all the grafted cells, since the majority of surviving recipients exhibited gradually increasing numbers of donor-type circulating erythrocytes. Furthermore, C57BL bone marrow incubated similarly with recipient liver caused 5 out of 5 (C57BL × 101) $F_1$ recipients to survive otherwise lethal X irradiation (900 r) and subsequently to show circulating donor-type erythrocytes (G. E. Cosgrove and R. A. Popp, 1959, unpublished data). It may be inferred, therefore, that incubation with recipient-type antigen *in vitro* decreased the ability of the implanted lymphoid cells to react immunologically against the recipient without decreasing the ability of stem cells to repopulate the marrow.

Although the inhibition of the graft's immunologic reactivity remains to be explained, inactivation of the grafted cells by a process like that postulated to account for the ontogenetic development of tolerance to self(7) is a conceivable mechanism. Other possibilities include changes in the host-graft relation, such as immunologic "paralysis," "adaptation," and "enhancement," which have been discussed previously in relation to bone marrow chimerism(8). That lymphoid cells may be destroyed by excess antigen has been suggested by Rich and Lewis(9) and Gorer and Boyse(6).

*Summary.* C57BL mouse spleen cells incubated with (C57BL × 101)$F_1$ liver homogenate killed only 17% of sublethally irradiated (C57BL × 101)$F_1$ recipients, whereas when incubated with C57BL liver homogenate or injected without incubation such cells killed 100% of sublethally irradiated (C57BL × 101)$F_1$ recipients within 35 days. Although the immunologically inactivated spleen cells showed a decreased ability to kill, circulating donor-type erythrocytes were found in surviving recipients, indicating repopulation of the bone marrow by surviving graft-derived stem cells. From this, it is inferred that immunologic inactivation did not result from nonspecific destruction of all types of implanted cells.

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# Effect of Epinephrine on Glucose Uptake and Glycerol Release by Adipose Tissue *in vitro*.* (25306)

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Available information suggests an inverse relationship between carbohydrate utilization and release of free fatty|| acids by adipose tissue. Decreased glucose metabolism (fasting, diabetes mellitus) is generally accompanied by increased free fatty acid release, and conversely, increased glucose metabolism (glucose feeding, insulin administration) by decreased release(1-5). *In vitro* addition of epinephrine to adipose tissue, however, results in accelerated free fatty acid release concomitant with increased rate of glucose assimilation. In addition, there is increased release of free glycerol from adipose tissue under these conditions. This report demonstrates that carbohydrate utilization and free fatty acid release are not necessarily linked, and suggests that rate of lipolysis may be regulated independently.

*Materials and methods.* Adipose tissue fragments from epididymal fat pads of normal fed rats (Wistar strain obtained from Albino Farms or Harvard Biological Labs) were incubated in Krebs-Ringer bicarbonate buffer containing 4% human serum albumin (obtained from Protein Fn., through courtesy of Dr. R. J. Pennell). Glucose concentration was 5 mM/L and that of free fatty acids bound to the albumin approximately 0.8 mM/L. For analysis of hormonal effects, tissues from the same animal were always used as controls. Incubations were otherwise carried out as previously described(6,7,8). At the end of incubation, medium glucose was determined by method of Nelson(9), after precipitation of proteins with $ZnSO_4$ and $Ba(OH)_2$. For glycerol determinations, an aliquot of the supernatant fluid was oxidized with periodic acid(10), and the formaldehyde thus generated was determined with chromotropic acid(11). That the formaldehyde formed in excess of amount derived from glucose could be taken to measure glycerol was shown by submitting an aliquot of supernatant fluid to paper chromatography. Using n-butanol-acetic acid-water (4:1:5) and n-butanol-acetic acid-water-conc. HCl (20:5:25:1) as solvents and lead tetraacetate as location reagent, 3 spots could be identified, cor-

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[||] Free fatty acid = nonesterified fatty acid (NEFA) or unesterified fatty acid (UFA).

TABLE I. Effects of Insulin and Epinephrine on Adipose Tissue Metabolism *In Vitro*. Values in $\mu$moles/100 mg wet weight adipose tissue/3 hours incubation. Mean of 9 experiments (standard errors in parentheses).

| | Glucose uptake | Free fatty acid release* | Glycerol release |
|---|---|---|---|
| Control | .47 (.07) | −.31 (.10) | .49 (.13) |
| Insulin (.1 unit/ml) | 1.99 (.13) | −.43 (.10) | .35 (.13) |
| Epinephrine ($10^{-4}$M) | 1.05 (.09) | 1.34 (.32) | 2.21 (.21) |

* Minus sign signifies uptake.

responding to mobilities of glucose, free glycerol and lactic acid(12). In experiments with labeled glucose, chromatography of a deproteinized aliquot of the medium after incubation, and scanning of strips with an open window proportional flow counter (Baird Atomic), demonstrated radioactivity peaks associated with the same spots. Free fatty acids in the medium were determined according to Gordon's method(13).

*Results.* Nine experiments are summarized in the Table, showing effects of insulin (0.1 unit/ml, using a highly purified crystalline preparation obtained through the courtesy of Dr. W. R. Kirtley) and epinephrine ($10^{-4}$ M) on glucose uptake and on exchange of free fatty acids between tissue and incubating medium. Also shown is the amount of free glycerol released by tissue under same conditions. Whereas insulin and epinephrine both increase glucose uptake, their effects on release of fatty acids are opposite. Insulin favors uptake of fatty acids, epinephrine favors their release. Likewise, insulin slightly decreases amount of free glycerol released, whereas epinephrine has a strong stimulatory effect upon this release.

*Discussion.* The effect of epinephrine on glucose uptake by adipose tissue was unexpected. One might rather have anticipated that epinephrine increases release of free fatty acids by diminishing availability of exogenous glucose to the tissue. The inhibitory effect of epinephrine on peripheral glucose utilization *in vivo*(14) and on glucose uptake of diaphragm muscle incubated *in vitro*(15) is indeed well documented and has been attributed to its glycogenolytic effect, with secondary accumulation of glucose-6-phosphate (16), a known inhibitor of hexokinase(17,18). Absence of an inhibitory effect of epinephrine on glucose uptake by adipose tissue may therefore be related to the barely detectable concentrations of glycogen found in adipose tissue from normal fed animals(19). Even so, the only well demonstrated effect of epinephrine at the cellular level, increased phosphorylase activity(20,21), provides no adequate explanation for epinephrine-induced increase in glucose uptake in adipose tissue. It is interesting that under certain conditions epinephrine can also increase glucose uptake of muscle(22).

Whatever the mechanism of this effect of epinephrine on adipose tissue, the fact that insulin and epinephrine both increase glucose uptake, yet have opposite effects on release of free fatty acids, has to be explained. Recently, epinephrine has been shown to stimulate several-fold the incorporation of glucose-$C^{14}$ into glyceride-glycerol by adipose tissue *in vitro*(23). Since net synthesis of glycerides presumably does not occur under these conditions, these findings suggest accelerated breakdown and resynthesis of glycerides, *i.e.* accelerated turnover. Furthermore, it would appear that glycerolphosphate newly synthesized from glucose is utilized for the esterification reactions, rather than free (unlabeled) glycerol resulting from the complete breakdown of triglycerides. Experiments by Shapiro(24), confirmed in our laboratory, have shown little utilization of exogenous labeled free glycerol, when compared to utilization of other substrates such as glucose or pyruvate. It is therefore not surprising to find a release of free glycerol by the control tissue, presumably reflecting lipolytic activity, even in the absence of hormonal stimulation. The increased release of free glycerol resulting from presence of epinephrine suggests an acceleration of this process. Furthermore, it is conceivable that accelerated lipolysis calls for accelerated re-esterification and thus for increased glycerolphosphate synthesis from glucose.

*Summary.* Epinephrine increases glucose uptake of adipose tissue incubated *in vitro*. This effect is common to both insulin and epinephrine. However, epinephrine increases and insulin decreases release of free fatty

acids. It would appear that the effect of epinephrine on mobilization of free fatty acids is not mediated by a decrease in carbohydrate availability, but possibly by increased rate of glyceride breakdown.

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## Antibody Response to Heterologous Protein in Rabbits of Varying Maturity.* (25307)

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Injection of a variety of heterologous proteins into the neonatal animal induces a state of immunological tolerance(1,2) which is of finite duration related to the amount injected at birth. The period of susceptibility to induction of tolerance to 100 mg bovine serum albumin in the rabbit has been found to end by 15-17 days of age(1). However, a state of immunological unresponsiveness similar in many respects to that induced at birth has been produced by repeated injections of very large amounts of heterologous proteins into adult rabbits(3). Calculations from available data do not entirely rule out the possibility that the difference in the adult and newborn response to heterologous protein may be a quantitative rather than a qualitative one. The 5-7 fold increase in body weight of rabbits during the first three weeks of age could conceivably reduce the concentration of injected antigen below a critical level. These experiments were designed to re-examine the immunological response of rabbits of varying maturity to weight-adjusted amounts of a relatively pure heterologous protein. The data add further evidence that a qualitative defect in immunological capacity exists in the neonatal rabbit.

*Procedure and methods.* New Zealand

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TABLE I. Effect of Weight Adjusted Amounts of BSA on Immune Response in Rabbits of Varying Maturity.*

| Litter No. | Age at initial inj., days | Avg wt of animals at initial inj., g | Anti BSA produced after | | Interpretation§ | |
|---|---|---|---|---|---|---|
| | | | Initial inj. | Challenging inj. | Tolerance induced | Immune response |
| 1 | 2 | 54 | 0/3† | ‡ | 7/7 (100%) | 0/7 |
| 2 | 8 | 141 | 0/4 | 0/4 | | |
| 3 | 14 | 170 | 0/8 | 0/5 | 12/15 (80%) | 3/15 |
| 4 | 14 | 221 | 0/4 | 2/4 | | |
| 5 | 15 | 190 | ‡ | 1/6 | | |
| 6 | 21 | 352 | 0/2 | 2/2 | 4/15 (27%) | 11/15 |
| 7 | 21 | 220 | 1/6 | 2/6 | | |
| 8 | 22 | 170 | 7/9 | 7/9 | | |
| 9 | 28 | 426 | 4/5 | 4/4 | 0/12 | 12/12 |
| 10 | 30 | 524 | 8/8 | 8/8 | | |

* Litters of rabbits were given 200 mg BSA/kg I.P. at ages indicated and bled serially for determination of presence of BSA or anti-BSA as precipitins in serum. At 10 wk of age the challenging inj., 20 mg BSA/kg was given I.V. followed by similar determinations on serial serum specimens.

† Denominator = No. in litter at time of bleeding. Numerator = No. with anti-BSA in serum.

‡ Not determined or not rechallenged.

§ Tolerance indicated by prolonged clearance of initial and challenging inj. *and* failure to produce detectable anti-BSA after antigen clearance. Immune response indicates production of detectable precipitating antibody.

white rabbits were used. Litters were kept with does from birth until weaning at 5 to 6 weeks of age. Procedures for identifying and handling neonatal rabbits have been previously described(1a). Blood samples were obtained for precipitin analysis by cardiac puncture. Crystalline Bovine Albumin (BSA) was obtained from Armour Labs, dissolved in 1% NaCl, sterilized by passage through Seitz filter, and analyzed for protein content by micro-Kjeldahl analysis. Rabbits were weighed and then given an injection calculated to total 200 mg BSA/kilo. Initial injections in each age group were given intraperitoneally. The amount was that known to produce tolerance lasting over 12 weeks in a majority of rabbits when given at birth(1a). Challenging injections of BSA were given intravenously through an ear vein in a dose of 20 mg/kg of body weight 10 weeks after initial injection. Following both initial and challenging injections, animals were bled at 5 day intervals and the serum assayed qualitatively for presence of BSA or of anti-BSA antibody by a capillary tube precipitation method(1a).

*Results.* Table I presents results in terms of production of precipitating antibody in serial bleedings following both first and second antigen injections. Litters 1, 2, and 3 had BSA present in their serum for 20 days or longer following initial injection and for 17 days or longer after the challenging injection (litters 2 and 3 only). These animals proved tolerant at the 10 week challenge as expected from results of previous studies. Of litters initially injected at 14 and 15 days of age, in only one litter (number 3) were all rabbits unresponsive at the challenge. In litters 4 and 5, 2 of 4 and 1 of 6 respectively, produced anti-BSA after the 10 week injection; whereas no antibody production was observed following neonatal injection.

Three litters were injected at 21 and 22 days of age. None of the rabbits in litter 6 but 1 of 6 in litter 7, and 7 of 9 in litter 8 had antibody in their serum following initial injection. Following the challenging injection, the majority in litters 6 and 8 produced antibody while ⅓ of litter 7 produced antibody.

Litters 9 and 10, who were initially injected on day 28 and 30 cleared antigen rapidly from their serum and 12 of 13 rabbits in both groups produced antibody following single exposure to antigen. The single rabbit in litter 9 which failed to produce antibody initially did not survive 10 weeks, and was not reinjected.

The experimental groups may conveniently be combined as shown in Table I, into 4 groups which responded to the antigen in a similar fashion. Those animals which showed prolonged clearance time of antigen injected both in the neonatal period and at 10 weeks and which showed no precipitating antibody after either injection, are considered to be tolerant(1a). Those producing circulating antibody after either injection are considered immune. By this grouping it is shown clearly that the amount of antigen injected initially, even though adjusted in amount for rapid growth of animals, was adequate to induce tolerance regularly only during the first 2 weeks of life, as previously shown for a single quantity of antigen. A marked qualitative change in animals' response to this heterologous protein, therefore, must have occurred between 14th day of life when 80% of the litters became tolerant and 21st day when only 27% failed to produce antibodies.

*Discussion.* Rabbits older than 28 days of age consistently produced antibodies to a single injection of BSA, while rabbits under 14 days of age handled this protein with no evidence of immune response. Rabbits between these 2 age groups responded variably, but most injected at 14 days of age were unresponsive while most 21-day-old animals had a primary immune response with production of precipitating antibody. These data again demonstrate that the period during which unresponsiveness can be induced is of finite length, consistent with a qualitative deficiency in the immune mechanism. These data also suggest strongly that different mechanisms probably underlie the model of unresponsiveness which is induced in adults by large amounts of antigen(3) and the type tolerance induced during the neonatal period.

*Summary.* Neonatal rabbits respond to a single injection of BSA in a dose of 200 mg/kilo body weight with antibody production when given at 28 days of age or older and are unresponsive when this weight-adjusted amount of BSA is given at 8 days of age or younger. At 14 and 21 days of age the response is varied and the majority injected at 14 days of age are unresponsive while most formed antibody when injected at 21 days of age.

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## Inhibition of Cleavage and Development of the Sea-Urchin Egg by 2-Desoxy-D-Glucose.* (25308)

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An analogue of glucose, 2-desoxy-D-glucose (2DG), has been shown to be a competitive inhibitor of glucose metabolism in yeast(1,2) and rat tissues(3-6). It is also known to retard cell-division in chick heart fibroblasts cultured *in vitro*(7), and in tumors(8-10), and budding in yeasts(2). In view of above work, it appeared to us that it would be of interest to investigate the action of this inhibitor on respiration and development of fertilized eggs of sea-urchins. Both glycolytic and respiratory metabolism are required for cleavage of these eggs, since development is blocked by fluoride and iodoacetate, as well as low $O_2$ tension, CO, and cyanide(11,12). At the same time, since in early development of the egg only endogenous nutrient is utilized, it has been difficult to specify what substrates are metabolized. Use of 2DG therefore should provide some information about the importance of glucose-metabolism to the dividing egg. The inhibitory effects of 2DG on cleavage, development and oxygen uptake of eggs from the sea-urchin *Strongylocentrotus purpuratus* are described here.

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*Methods.* Eggs were obtained from excised ovaries and washed several times in filtered sea water before use. Eggs were mixed 15 minutes after fertilization with equal volumes of a solution of 2DG in spot plates or salt cellars which were sealed and stored at 16-17°C ("dish" experiments). 2DG was prepared as a 1.0M stock solution in distilled water buffered at pH8 with 0.01M (final conc.) glycylglycine (gly.gly.)(13). The stock solution, which was approximately isosmotic with sea water, was diluted to desired concentration with 0.01M gly.gly. sea water at pH8. Control eggs were mixed with glucose solutions, prepared in the same manner as 2DG, or with gly.gly. sea water alone. In manometric experiments eggs were resuspended in 0.025 M gly.gly. sea water, pH 8, after the last washing. The diluent contained 100 units penicillin and 0.1 mg streptomycin/ml. Egg suspensions were added to Warburg vessels from 10 to 45 minutes after fertilization, and an appropriate volume of 2DG, glucose, or 0.01 M gly.gly. sea water was added from a side arm either at 15 minutes (during temperature equilibration or just before vessels were placed in bath) or at about 105 minutes after fertilization. Oxygen uptake was measured at 19.5°C by direct method with air as the gas phase.

*Results. Effect on cleavage and development.* 2DG was tested at concentrations of $10^{-5}$ to 0.5M, and the eggs observed 40 to 60 hours, by which time all control embryos were at gastrula stage. Concentrations from $10^{-3}$ to 0.5M invariably arrested development, but there were variations between experiments as to the stage at which development stopped. This seemed to be due to differences in susceptibility of eggs from different animals. A summary of results of these tests, with number of experiments in parenthesis, follows: $10^{-3}M$(5) and $10^{-2}M$(7) prevented eggs from gastrulating; eggs reached early (unhatched) or swimming blastula stage. *0.1 M*(6) delayed first cleavage, but eggs eventually cleaved and some went as far as blastula stage. 0.2 *M*(4) caused a greater delay in first cleavage and generally blocked development before eggs reached blastula stage. The inhibited eggs maintained their normal appearance for several hours, but there was usually no reversal of inhibition upon washing with sea-water. Glucose at $10^{-3}$ to 0.2M did not retard development; however, eggs in glucose (concentrations as low as $10^{-2}$M) developed somewhat abnormally at later stages. They formed blastulae and gastrulae with unusually thick walls and occasionally gastrulation was not completed.

TABLE I. Effect of 2DG and Glucose on $O_2$ Consumption of Eggs, in 8 Experiments.

| Time of addition of sugar (min. after fertilization) | Rate $O_2$ uptake of treated eggs 6 hr after sugar added (as % of control)<br>2DG conc., moles/l | | | | Glucose conc., moles/l | | |
|---|---|---|---|---|---|---|---|
| | .5 | .2 | .1 | .01 | .5 | .2 | .1 |
| 107 | | 7 | 17 | 38 | | 96 | 106 |
| 110 | | 24 | 28 | 71 | | 88 | 99 |
| 105 | 16 | 31 | | | 91 | 92 | |
| 105 | 30 | 48 | | | 100 | 101 | |
| 15 | 32 | 54 | | | 117 | | |
| 110 | 31 | 52 | | | | | |
| 15 | 15 | 37 | | | 93 | | |
| 105 | 17 | 29 | | | | | |
| 15 | | | 37 | | | 91 | |
| 15 | | | | 72 | | | |

Eggs in .5M 2DG cleaved only once or twice; .5M glucose did not appreciably affect rate of cleavage.

*Effect on oxygen uptake*: Manometric experiments were run for 7 to 10 hours, at which time the eggs were removed from flasks and their stage of development determined. Each concentration of 2DG tested ($10^{-2}$ to 0.5M) inhibited both oxygen uptake and development. The extent of inhibition of oxygen uptake is shown in Table I. Eggs in 0.1 and 0.2M, and in other experiments 0.5M, 2DG showed no rise in rate of oxygen uptake such as that found in normally developing controls. As control rate increased, the rate for treated eggs remained constant and eventually declined. The length of time required for the decline in rate to begin depended on concentration of 2DG. Oxygen uptake of eggs in $10^{-2}$M 2DG also lags as control rate increases, but nevertheless it increases above its original level before inhibition sets in. (In one experiment the curve for 0.2M was of this type.) Glucose had essentially no effect on oxygen uptake.

Inhibition of development was about the same as in the dish experiments. Eggs in $10^{-2}$M 2DG were one to 2 cleavages behind controls, which were at a late cleavage stage (about 256 cells) at the end of the experiments, while eggs in higher concentrations were more retarded.

There was a definite relationship between inhibition of oxygen uptake and inhibition of cleavage. The earlier the inhibition of oxygen uptake occurred, the greater was the retardation of cleavage.

*Glucose as a counteractant to 2DG*: One characteristic of competitive inhibition is that an excess of normal substrate will counteract the effect of the inhibitor. To determine whether inhibition caused by 2DG was competitive in nature, eggs were treated with mixtures of 2DG and glucose with the result that glucose almost completely counteracted the effect of 2DG on respiration (Table II). The delay in cleavage caused by 2DG was also offset by glucose. Addition of glucose after inhibition had begun, did not reverse the effect of 2DG on respiration or cleavage. In mixtures of 0.01M 2DG and 0.05M succinate, inhibition was as great as in 2DG alone; succinate did not alleviate the effect of 2DG.

TABLE II. Effect of Excess Glucose on Respiratory Inhibition of Developing Eggs by 2DG, in 3 Experiments.

| Rate of $O_2$ uptake 8 hr after addition of sugars, expressed as % of control rate in sea-water | |
|---|---|
| 2DG only, .01M | 2DG .01M + glucose .1M |
| 57 | 92 |
| 24 | 92 |
| 18 | 94 |

*Discussion.* Our experiments show that 2-DG inhibits cleavage and oxygen consumption of sea-urchin eggs. Cleavage is first slowed, then totally blocked by 2DG. Since the effect of 2DG is counteracted by excess glucose, it is tentatively concluded that 2DG competitively inhibits glucose metabolism of the eggs.

Sea-urchin eggs contain enzymes both for anaerobic glycolysis and the hexose monophosphate shunt(14). Possible sites on these pathways at which glucose metabolism may be inhibited are glucose → glucose-6-phosphate, since egg hexokinase phosphorylates both glucose and 2DG(15); glucose-6-phosphate → fructose-6-phosphate(16); and glucose-6-phosphate → 6-phosphogluconic acid, which is the first reaction of the shunt. The latter site is suggested, although it has not been demonstrated to be inhibited by 2DG, because glucose metabolism in sea-urchin eggs may proceed largely through the shunt(17,18).

It is also possible that phosphorylation of 2DG slows cleavage to some extent by reducing intracellular level of adenosinetriphosphate (ATP). 2DG-6-phosphate apparently is not metabolized(19), and the phosphorylation would therefore consume ATP without eventually leading to synthesis of more ATP.

2DG should be a useful tool in the study of egg metabolism and cleavage, although for the latter purpose the high concentrations required for rapid inhibition may be disadvantageous. The delay observed with low concentrations may be due to a slow uptake of 2DG by eggs, or it may be inherent in the mechanism of inhibition.

*Summary.* 2DG inhibits cleavage and oxygen consumption of sea-urchin eggs. High concentrations inhibit cleavage and oxygen uptake at early stages and low concentrations inhibit at later stages of development. The effect of 2DG was counteracted by excess glucose which suggests that 2DG affects cleavage by competitively inhibiting glucose metabolism.

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## Alkali-Soluble and Insoluble Collagen in Infant, Adult and Cirrhotic Livers.[*] (25309)

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Recent investigations emphasized the importance of the distinction between insoluble and soluble collagen, the latter being tentatively considered a precursor of insoluble collagen(1,2,3,4). The radioactive - isotope studies of Harkness indicated that alkali-soluble collagen rather than acid-soluble collagen is the true precursor(3). During evolution of experimental hepatic fibrosis produced by ethionine, the increase in alkali-soluble collagen precedes that of insoluble collagen and relative rate of increase of soluble collagen greatly exceeds that of the insoluble(5). A similar fractionation of collagen was performed, therefore, on human livers in which collagen was increasing either under physiologic circumstances, as in the infant, or under pathologic conditions, as in cirrhosis. Concentration of hydroxyproline as a measure of collagen was compared with histologic distribution of collagen and reticulum fibers.

*Materials and methods.* All livers were obtained at autopsy. The adult control group was derived from patients between ages 33 and 90, who died from diseases without known influence on hepatic connective tissue. The infant group comprised 7 premature newborn infants and 8 full-term infants, varying in age from 1 day to 9 months. The cirrhotic group consisted of 10 postnecrotic cirrhoses, one case

TABLE I. Alkali-Soluble and Insoluble Collagen in Infant, Adult and Cirrhotic Livers.

| | | Total liver | | Alkali-soluble fraction | | | Histological grading‡ | |
|---|---|---|---|---|---|---|---|---|
| | No. of cases | Hydroxy-proline mg %* | Collagen† mg %* | Hydroxy-proline mg % | Collagen mg % | Sol. collagen as % of total % | Collagen | Reticulum |
| Control adult | 6 | .376 ±.122§ | 2.80 | .039 ±.009 | .29 | 10.58 ± 2.62 | ++ | ++ |
| Infant | 15 | .155 ±.036 | 1.15 | .037 ±.013 | .27 | 24.10 ± 9.00 | + | + |
| Cirrhosis | 12 | 1.391 ±.590 | 10.37 | .313 ±.081 | 2.33 | 22.57 ± 5.90 | ++++ | +++++ |

* mg % of dry, defatted liver. † Collagen content calculated on the assumption that both soluble and insoluble collagen contain 13.4% hydroxyproline. ‡ Collagen and reticulum were graded histologically 1+ to 5+. § Stand. dev.

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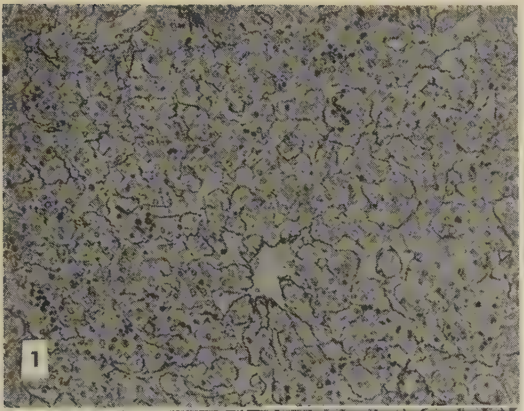
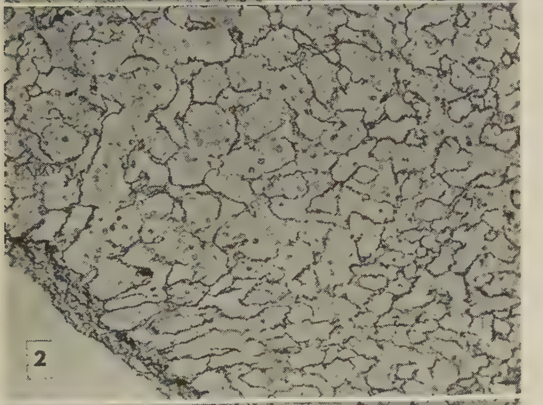
[†] Fellow of Dazian Fn.





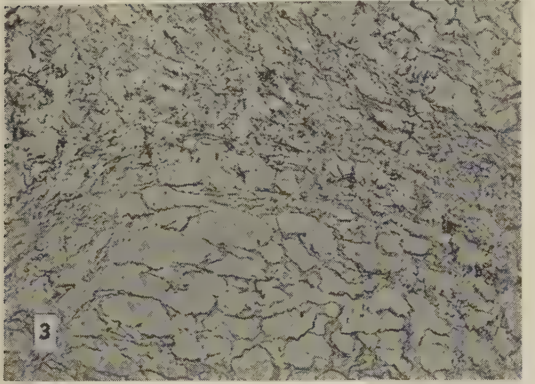


FIG. 1. Liver of premature infant. Hydroxyproline concentration .120 mg/100 mg dried, defatted liver. Alkali-soluble collagen 28.5%. Gomori silver impregnation, 120×.

FIG. 2. Liver of a control adult. Hydroxyproline concentration .348/100 mg dried, defatted liver. Alkali-soluble collagen 10.7%. Gomori silver impregnation, 120×.

FIG. 3. Liver of adult with postnecrotic cirrhosis. Hydroxyproline concentration 1.970 mg/100 mg dried, defatted liver. Alkali-soluble collagen 21.4%. Gomori silver impregnation, 120×.

of hemochromatosis, and one of myeloid metaplasia with excessive fibrosis but without cirrhotic transformation. Purification and partition for chemical analysis were carried out according to method previously described(5). For extraction of soluble collagen, 0.1 N NaOH was employed. The hydroxyproline content of dry defatted liver tissue was determined by modification of the method of Neuman and Logan(6). For histologic study, $5\mu$-thick sections were stained with H and E or chromatrope –2R aniline blue(7), or subjected to Gomori's silver impregnation. Amount of collagen, as reflected by aniline blue staining and that of reticulum as indicated by silver impregnation, were arbitrarily graded 1 to 5 +.

*Results.* The defatted and dehydrated portion of the control (normal adult) liver contained approximately 0.4% hydroxyproline, which corresponds to 3% collagen (using factor of 7.46(6)). In cirrhotic liver, total hydroxyproline concentration was approximately 3.7 times greater than that of the control, without any overlap. In contrast, the infant liver contained only about 40% of total hydroxyproline concentration of the control liver. There was, however, approximately 8 times as much hydroxyproline in the soluble fraction of cirrhotic liver as in the control liver, whereas hydroxyproline concentration in the soluble fraction of control and infant liver was virtually the same. In the control liver, the hydroxyproline of the alkali-soluble fraction comprised about 10% of total hydroxyproline, while in both infant and cirrhotic livers, this fraction accounted for approximately 25% of total hydroxyproline (Table I). No marked differences were found between livers of premature and full-term infants. Livers of premature infants had a mean total hydroxyproline concentration of 0.128 g/100 g of dry defatted liver with 26.7% in the soluble fraction, while the group of full-term infants had a mean total hydroxyproline concentration of 0.179 g/100 g of dry defatted liver with 21.8% in the soluble fraction.

Histologically, both collagenous and reticulum fibers were significantly increased in cirrhosis. In infant livers, considerably less reticulum was found than in control adults (Fig. 1, 2, 3). Liver cells were frequently arranged in more than one cell-thick plates, and therefore, the sinusoidal surface on which the reticulum fibers were aligned was relatively less extensive. Moreover, along the surfaces were fewer fibers than in the adult liver. The difference in amount of stainable collagen fibers between infants and control adults was in great measure accounted for by the lesser amount in the portal tracts of infant livers. No reliable differences in the listed histologic characteristics between premature infants and babies could be demonstrated.

*Discussion.* The increase in total collagen concentration in cirrhosis, as measured by hydroxyproline concentration, has been repeatedly demonstrated(8,9). The range found in our study is consistent with that reported by Kent *et al.*(9). In the infant liver, collagen concentration is considerably lower, corresponding to the range reported for adult rat (5,9). Lower concentration of hydroxyproline is reflected in the amounts both of stainable collagen and of silver impregnated reticulum, which according to electron microscopic studies appear to be identical. The relatively lower collagen concentration in the infant liver is a reflection of the more intensive growth of parenchyma as compared to stroma. This situation also obtains in hepatic regeneration after hepatectomy(10) and is reflected microscopically in plates that are several cells thick. To this quantitative difference must be added a qualitative alteration of the collagen of the human infant liver, the proportion of soluble collagen being much higher. The fact that the ratio between soluble and total collagen in infant liver is of similar magnitude as in the cirrhotic liver, although the latter contains almost 10 times as much total collagen, tends to confirm the assumption of Harkness *et al.*(3) that the alkali-soluble collagen is a precursor of the insoluble collagen, and is evidence that this is independent of the conditions under which the fibers are formed. Increased amounts of soluble collagen were previously reported in uterus, tail tendon, aorta, and skin of young rats(11) and in lathyritic chick embryo(12).

*Summary.* Concentration of hydroxyproline in the liver, as a measure of collagen, is lower in human infants than in adults, and histologically-stainable collagen and silver impregnated reticulum fibers are fewer. While in cirrhosis, total collagen concentration is 10 times greater than in infant livers, in both cirrhotic and infant livers the ratio of alkali-soluble collagen to total collagen is similar and considerably higher than in control adults. This suggests that formation of new collagen fibers, under both physiological and pathological circumstances, is associated with a relative increase of alkali-soluble collagen.

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# Bacterial Polysaccharide, Cortisol, and Acute Leukemia in the Rat.* (25310)

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Murphy and Sturm(1) described a transplantable lymphosarcoma originally induced by 1, 2, 5, 6 dibenzanthracene in the rat. When the tumor cells were injected subcutaneously, a lymphosarcoma developed at that site, while their intraperitoneal injection resulted in rapidly fatal leukemia. Like other malignant neoplasms in rodents(2-6), the Murphy-Sturm lymphosarcoma undergoes necrosis following injection of polysaccharide complexes of Gram-negative bacteria. In previous studies(7,8) necrosis and regression of the Murphy-Sturm lymphosarcoma has been induced by *Klebsiella pneumoniae* polysaccharide. The present study concerns effects of *K. pneumoniae* polysaccharide in the Murphy-Sturm leukemia, inhibition of anti-leukemic effect of bacterial polysaccharide by cortisol administration, and tumor resistance following successful treatment of leukemic rats.

*Materials and methods.* Young, male Sprague-Dawley (Holtzman) rats, weighing 125-135 g, were maintained on Purina laboratory chow, lettuce and water. Except for normal controls, each rat received approximately 100,000 cells from a Murphy-Sturm lymphosarcoma, suspended in 0.85% NaCl and culturally free of pleuropneumonia-like organisms and bacteria. Part of the animals received tumor cells intraperitoneally and the remainder, intravenously. Body weights and peripheral blood leukocyte counts were determined daily and erythrocyte counts and hematocrits were done at various intervals during experiment. Haptenic polysaccharide, non-lethal in control rats(9), was prepared by alkaline extraction and ethanol precipitation from *Klebsiella pneumoniae* as previously described(10). Except for leukemic controls, rats from both intraperitoneally and intravenously implanted groups were given 4 daily consecutive injections by tail vein, 0.1 mg/100 g body weight/injection, of the polysaccharide prepared as 0.1% solution in 0.85% NaCl. Animals in the intraperitoneally implanted group were given the first polysaccharide injection when peripheral blood contained lymphoblasts and total leukocyte count exceeded 20,000/mm$^3$. Intravenously implanted rats received the initial polysaccharide injection either upon first decline in growth rate or 1 or 2 days later upon first elevation of white blood cells. Beginning 2 days prior to the polysaccharide injections daily subcutaneous injections of cortisol acetate, 2.5 mg/100 g body weight/day, were given to part of the rats in intravenously and intraperitoneally implanted groups and at about the same time (5 to 8 days after tumor cell implantation) in the leukemic control rats. Cortisol administration was continued until early death of leukemic rats or for 1 week after last polysaccharide injection in surviving animals. To evaluate the effects upon body weight, one group of normal rats was given daily injections of cortisol acetate and another group, the 4 daily injections of polysaccharide. One month after recovery from leukemia, part of the polysaccharide-treated rats were reinjected by tail vein with a suspension of lymphosarcoma cells and body weight and white blood counts determined every few days for 3 months. Autopsies were performed and microsections of various tissues prepared from animals after polysaccharide treatment and from untreated leukemic rats 1, 3, 5, 7 and 9 days after implantation of tumor cells. Smears of femoral bone marrow were prepared with Wright's stain.

*Results.* Leukemia developed in all untreated animals receiving lymphosarcoma cells by either intraperitoneal or intravenous route. No spontaneous remissions were observed and all untreated animals died with evidence of leukemia, anemia and sometimes with hemorrhages in and about the eyes. Cessation of normal growth and decline in body weight regularly preceded by 1 or 2 days the elevation of leukocytes in peripheral blood (Fig. 1, 2). Seven days after injection of tumor cells,

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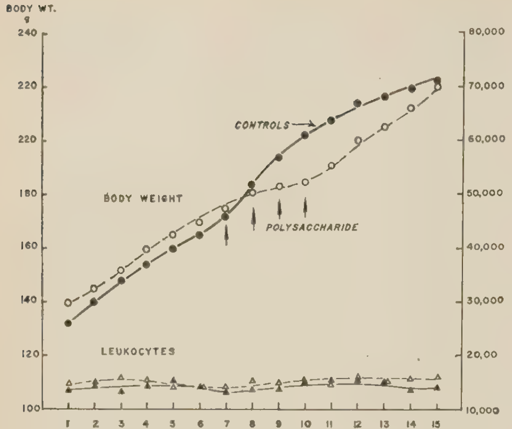

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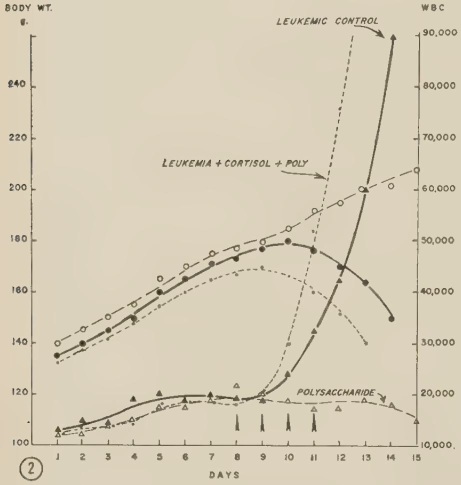

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FIG. 1. Effect of 4 injections of *K. pneumoniae* polysaccharide (0.1 mg/100 g body wt) upon body wt and leukocytes of peripheral blood in non-leukemic, young, growing rats (leukocyte counts 20 hr after inj. of bacterial polysaccharide). Normal controls: body wt ●——●, WBC ▲——▲. Polysaccharide injected: body wt ○– –○, WBC △– –△.

FIG. 2. Absence of anti-leukemic effect of bacterial polysaccharide in cortisol-treated rats. *K. pneumoniae* polysaccharide inj. in rats beginning 8 days after intrav. transplantation of tumor cells and with leukemic infiltration of marrow, liver and spleen but without elevation in white blood count. Leukemic controls: body wt ●——●, WBC ▲——▲. Leukemia + polysaccharide: body wt ○– –○, WBC △– –△. Leukemia + polysaccharide + cortisol: body wt ● - - - ●, WBC ▲ - - - ▲.

body weight no longer increased and histologic sections revealed infiltration of large lymphoblastic cells into bone marrow, liver and spleen. The rats died within 1 to 3 days following onset of leukocytosis. During earliest phase of leukocytic increase, mature cells of granulocytic series were prominent but subsequently the large lymphoblasts predominated, constituting 85-97% of cells in blood, femoral marrow, and markedly infiltrating liver and spleen. Animals dying with leukemia following intravenous implantation were similar to those after intraperitoneal implantation except that some of the latter had jaundice and bile duct obstruction due to tumor tissue about the porta hepatis and small bowel mesentery.

Cortisol administration to control rats did not alter their growth rate. Cortisol did not modify the time of appearance of leukemia, but rats so treated died with marked elevation in white blood cells and pronounced anemia about one day sooner than the untreated leukemic animals.

The 4 injections of bacterial polysaccharide slightly decreased the weight increase in healthy, growing rats (Fig. 1); none of the rats died. When polysaccharide injections were given to leukemic rats with marked elevation of their white blood count, 60% died after first or second injection, while the remaining 40% showed a transient decrease in white blood count but died with leukemia 4 to 5 days after the rats in the untreated leukemic group. When the polysaccharide was injected in rats in the aleukemic phase of their acute disease, *i.e.*, with a decreased growth rate and leukemic infiltration of tissue but without change in leukocytes of peripheral blood, 80-90% of the animals recovered and showed no subsequent evidence of the disease (Fig. 2, Table I). Half of the recovered animals were reinjected with tumor cells but failed to develop leukemia or lymphosarcoma.

The anti-leukemic effects of bacterial polysaccharide were markedly reduced by daily injections of cortisol (Table I).

*Discussion.* The present data indicate that

TABLE I. Effect of *K. pneumoniae* Polysaccharide and Cortisol upon Survival of Leukemic Rats (10 Rats/Group).

| | Treatment | | | |
|---|---|---|---|---|
| Route of inj. of tumor cells | Control | Cortisol | Cortisol + poly. | Polysac. |
| Intraper. | 0 | 0 | 10% | 80% |
| Intrav. | 0 | 0 | 0 | 90% |

the Murphy-Sturm leukemia, like the lymphosarcoma produced by the same cells(7), regresses upon injection of polysaccharide from *K. pneumoniae*. There are other similarities between the Murphy-Sturm leukemia of the rat and experimental malignant tumors in rodents: (a) the enhanced lethal effect of bacterial products in animals with large tumors(7) and with advanced leukemia, (b) decreased therapeutic effectiveness of bacterial polysaccharide in rats with large tumors and in advanced leukemia, (c) resistance or "immunity" to tumor cells following regression of the lymphosarcoma(11) and of the leukemia, (d) inhibition of anti-leukemic and tumor-necrotizing effects (unpublished studies) of bacterial polysaccharide by cortisol administration. Studies by Stoerk(11) and unpublished studies by us indicate that resistance of rats with regressed tumors to reinoculation disappears with cortisone administration. Corticosteroid administration also permits transplantation of tumor to heterologous hosts(12).

The various considerations above may be correlated by postulating an indirect mechanism of tumor injury by bacterial polysaccharide, wherein the opposing effects of cortisol and polysaccharide, as well as acquired tumor resistance, are mediated through non-neoplastic tissues. Even the simplest scheme includes lymphoid tissue, cytotoxins, plasma proteins, tumor tissue, pituitary and adrenal. Lymphoid tissue is adversely affected by corticosteroids(13) and the lymphocyte is implicated in host resistance to tumor transplants(12,14, 15). Tumor necrotizing(16) and ACTH-releasing(17) properties as well as other effects of bacterial products are diminished by plasma proteins. Bacterial polysaccharide transiently increases adrenal corticosteroid production in the guinea pig(18) and probably in the rat(19) thus implying that the injurious effects upon tumor tissue are opposed, at least transiently, by the effects upon the pituitary and adrenal. Maintenance of adrenal weight with reduction in splenic and thymus weight in rats with large tumors(7) may reflect accompanying functional changes in these tissues, thereby explaining why the bacterial polysaccharide is not only more lethal but, in surviving animals, is less effective in inducing regression of the neoplastic process in rats with large tumors or advanced acute leukemia.

The various aspects briefly outlined above are only suggestive of the complex inter-relationships requiring experimental evaluation before the mechanism of induced tumor-necrosis is understood.

*Summary.* *K. pneumoniae* polysaccharide, injected intravenously, cures rats with leukemia due to Murphy-Sturm lymphosarcoma cells. Cortisol administration markedly inhibits the anti-leukemic effect of the bacterial polysaccharide. Resistance to tumor cells follows the polysaccharide-induced recovery from leukemia.

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# ATP and the Conversion of Mevalonic Acid to Cholesterol and Precursors By Liver Homogenates.* (25311)

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Inability of whole rat liver homogenates preincubated with ribonuclease (RNAse) to convert mevalonic acid (MVA) into non-saponifiable material (NSF) is associated with absence of ATP in these homogenates(1,2). Control homogenates preincubated without RNAse actively convert MVA into NSF and contain relatively large amounts of ATP. Homogenates preincubated with RNAse and heated liver extract from which ATP was removed by treatment with anion exchange resin contain ATP following preincubation and similarly convert the precursor into NSF. It has been concluded that the factor of liver extract essential for utilization of MVA by RNAse-treated homogenates is concerned with maintenance in these preparations of a functional level of ATP(3,4). Under conditions studied the conversion of MVA to NSF appears to be a direct function of ATP level existing in a homogenate at the time it is supplemented with MVA. This communication is concerned with the effect of ATP added to homogenates either before or after preincubation with or without RNAse under conditions of an aerobic or anaerobic environment.

*Methods and materials.* Experimental procedures involved in biosynthetic studies have been described in detail(5). In brief, liver from young rats is homogenized with a loose homogenizer and centrifuged at 200 xg for 3 min. to remove connective tissue. The top layer together with a loosely packed intermediate layer are decanted to yield a "200 xg supernatant fraction." An aliquot of this fraction is centrifuged at 9000 xg for 10 min. to yield a "9000 xg supernatant fraction" considered free of tissue fragments. 5 ml aliquots of the 200 xg or 9000 xg supernatant fractions fortified with DPN (1 mg) are preincubated (30 min.) in 125 ml Erlenmeyer flasks with and without ribonuclease (10 mg) in total volume of 10 ml. The present experiments differ from those previously described in that all flasks are supplemented with sodium succinate at 0.05 M. Supplementation with this substrate reduces the incidence of experiments where no incorporation of MVA occurs even in control flasks. ATP (10 mg unless otherwise indicated) where studied was added prior to or following the preincubation period. Following preincubation 1 ml of MVA-2-$C^{14}$ solution was added to each flask and the flasks reincubated (3 hr.) for a determination of the capacity of treated homogenates to synthesize NSF from labelled precursor. Flasks were flushed with either oxygen or nitrogen prior to preincubation and again at time of MVA addition. Following incubation of flasks containing added MVA-2-$C^{14}$ the contents were saponified, extracted with petroleum ether, the extract dried with sodium sulfate, filtered, evaporated, taken up in scintillation mixture and counted(5). The data presented are in terms of total counts recorded/incubation flask.

*Results.* Fig. 1 shows the effect on biosynthesis of labelled NSF of varying amounts of ATP added to homogenates following a preincubation period with and without RNAse in an atmosphere of oxygen. Under these conditions ATP is inhibitory to MVA utilization in aliquots of the homogenate preincubated without RNAse but it is somewhat stimulatory to MVA utilization in similar aliquots preincubated with RNAse. Experiments involving addition of ATP to homogenates prior to preincubation period yielded essentially similar results except that the magnitude of "stimulation or inhibition" was generally less.

Fig. 2 A shows the effect of ATP, RNAse, and aerobic *vs.* anaerobic environment on biosynthesis of labelled NSF from MVA-2-$C^{14}$ by a 200 xg and a 9000 xg supernatant fraction of liver homogenate. In this experiment, A, ATP was added to flasks prior to preincubation period. As reported previously, in an

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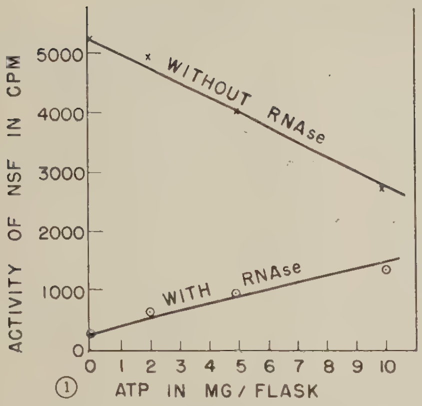
* Supported by research grants from Nat. Science Fn. and Nat. Inst. Health and by funds made available through State Univ. of N. Y.



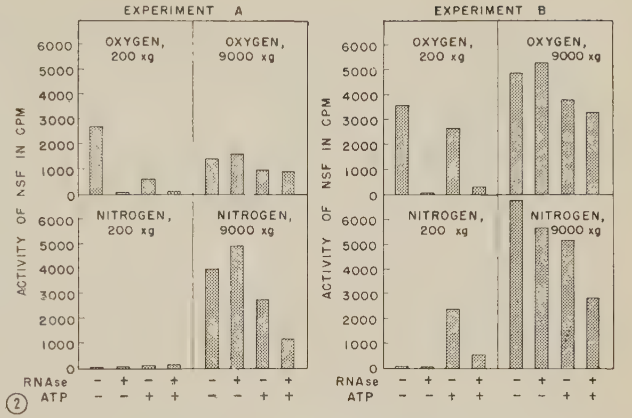


FIG. 1. Effect of ATP on biosynthesis of NSF from MVA by a 200 xg supernatant fraction of rat liver following preincubation with or without RNAse.

FIG. 2. Influence of ATP on biosynthesis of NSF from MVA by 200 xg and 9000 xg supernatant fractions of rat liver homogenate under aerobic and anaerobic conditions following preincubation with or without added RNAse. A. ATP added prior to preincubation. B. ATP added following preincubation.

oxygen atmosphere and with an essentially whole homogenate preincubation with RNAse abolishes the capacity of the preparation to convert MVA to NSF. ATP is inhibitory in the absence of RNAse. With a 9000 xg supernatant fraction of liver homogenate incubated under oxygen no inhibition of MVA incorporation is obtained by preincubation with RNAse as has been reported previously. ATP is somewhat inhibitory and its effect is not influenced by the presence of RNAse. Under nitrogen there is essentially no incorporation of MVA-2-$C^{14}$ into labelled NSF by a 200 xg supernatant preparation of liver homogenate and in this experiment there was no effect of added ATP although in other experiments involving ATP added prior to preincubation some stimulation of incorporation has been observed. In contrast, a 9000 xg supernatant fraction readily incorporates MVA into NSF under nitrogen. Preincubation with RNAse was without effect on the system. ATP was inhibitory and this inhibition seemed to be greater in the presence of RNAse.

Fig. 2 B summarizes the results of similar experiments described in Fig. 2 A except that here ATP was added to the homogenates after the preincubation period at time of MVA-2-$C^{14}$ addition. The results are essentially the same except that under nitrogen ATP is definitely stimulatory to incorporation with a 200 xg preparation.

*Discussion.* The data are readily interpretable in terms of extent to which a source of ATP is available for phosphorylation of MVA. The amount of ATP existing at any particular time in a homogenate may be considered as the steady state level of that originating largely from oxidative phosphorylation against that undergoing hydrolysis by various ATPases. Thus with a 200 xg fraction incorporation of MVA into NSF takes place in an atmosphere of oxygen because oxidative phosphorylation is able to keep ahead of the ATPases. On the other hand, with a 200 xg fraction incorporation of MVA into NSF does not take place in an atmosphere of nitrogen because in absence of oxidative phosphorylation a positive balance of ATP is not maintained in the face of the same level of ATPases. It is under these latter conditions that stimulation of biosynthesis by added ATP is sometimes observed particularly when ATP is added along with the MVA after the preincubation period.

A 9000 xg fraction incorporates MVA into NSF under either aerobic or anaerobic conditions because in the relative absence of ATPases which are largely sedimented in going from a 200 xg to a 9000 xg fraction the demands for ATP can be met by that present in the homogenate as well as by that formed by anaerobic substrate phosphorylation.

RNAse inhibition of MVA utilization occurs only with the essentially whole homogenate incubating under oxygen. Such RNAse-treated homogenates are devoid of ATP and evidence has been presented that the factor of liver extract that prevents or reverses RNAse inhibition is concerned with the maintenance of an adequate level of ATP. It would appear that the liver extract factor is involved either as a cofactor in the generation of ATP or as an inhibitor of ATP hydrolysis.

*Summary.* The influence of ATP on biosynthesis of cholesterol and precursors from mevalonic acid has been studied with relatively crude (200 xg supernatant) and more refined (9000 xg supernatant) fractions of rat liver homogenate under aerobic and anaerobic conditions following preincubation with or without added ribonuclease. Mevalonic acid incorporation occurred under conditions favorable for oxidative phosphorylation or under conditions where the ATPase of tissue fragments had been removed by centrifugation (9000 xg). Ribonuclease inhibition of mevalonic acid utilization occurred only under oxygen and in the presence of tissue fragments relatively rich in ATPase.

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# Serum, Bile and Liver Total Cholesterol of Laboratory Animals, Toads and Frogs.* (25312)

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There is much information in the literature on serum and tissue cholesterol concentrations of laboratory animals. Values reported vary a great deal. The differences can be attributed in part to the various methods of extraction and estimation procedures used. During the past few years a saponification-extraction procedure has been used in this laboratory to determine serum and tissue total cholesterol. The color is developed with the ferric chloride reaction. This procedure is adapted to determine an amount as small as a few μg of total cholesterol per sample(1). This communication reports serum, bile, and liver total cholesterol levels of 9 species of laboratory animals most commonly used in this laboratory and of 2 species of toads and 2 species of frogs.

*Material and methods.* The animal and amphibian species, body weight, sex, source of blood, and anesthetics used are compiled in Table I. All animals were from our animal stock house. They were kept on ordinary laboratory chow made by Purina for the respective species. The toads and frogs had been kept in the refrigerator. Bile samples were collected from the gall bladder of monkeys, dogs, cats, rabbits, guinea pigs, toads and frogs. Hepatic bile was collected from rats through cannulation of the common bile duct with polyethylene tubing. Total cholesterol was determined on an aliquot of undiluted serum, bile, and liver digested with potassium hydroxide(1). Samples were usually collected and kept in the refrigerator and determinations were made within a few days. Storage of samples in the refrigerator for a few days did not seem to affect total cholesterol concentrations.

*Results.* The serum, bile, and liver total cholesterol concentrations are summarized in Table II. There is no direct relationship between total cholesterol concentrations and body weight in the various species studied, nor is there a close relationship between se-

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* The authors are indebted to members of these laboratories who assisted with collection of specimens used.

TABLE I. Animals, Body Weight, Sex, Source of Blood and Anesthetics Used.

| Animals | Body wt range, kg | Sex | Source of blood | Anesthetics |
|---|---|---|---|---|
| Rhesus monkey | 2 – 3 | Mix | Femoral artery | Na amytal |
| Mongrel dog | 8 –15 | " | Femoral vein | " |
| " cat | 1.5 – 2 | " | " " | Chloralose |
| Albino rabbit | 2.5 – 3 | ♀ | Heart | None |
| Guinea pig | .650– .850 | ♀ | " | " |
| Golden hamster | .150– .200 | Mix | " | Ether |
| Albino rat | .100– .150 | ♀ | Decapitation | Na pentobarbital |
| " mouse | .015– .020 | ♀ | " | None |
| 'Barn' pigeon | .240– .340 | Mix | Heart | " |
| Nebulous toad *Bufo valliceps* | .016– .032 | ♀ | " | Pithed |
| Toad *Bufo fowleri* | .012– .020 | ♀ | " | " |
| Bull frog *Rana catesbeiana* | .195– .545 | Mix | " | " |
| Small frog *Rana pipiens* | .028– .032 | ♂ | " | " |

rum, bile, and liver total cholesterols. Serum total cholesterol concentration of the dog is higher than that of the monkey and the cat. On the other hand, the monkey has a higher concentration of total cholesterol in the bile and the liver.

Among the 5 species of rodents studied, total cholesterol concentrations in the serum and the liver are highest in hamsters and mice, lowest in rabbits and guinea pigs, and those of rats in between. Rabbits excrete relatively large amounts of cholesterol in the bile, while the bile total cholesterol level of guinea pigs is very low.

Pigeons, the only species of fowl studied, have the highest concentration of serum and

TABLE II. Serum, Bile and Liver Total Cholesterol Concentrations.

| Animals | Serum, mg % | Bile, mg % | Liver, mg/100 g wet wt |
|---|---|---|---|
| Monkey | 111.7 ± 1.8* 75.0–150.0†<br>(20) | 272.3 ±26.2 125.0–500.0<br>(17) | 284.4 ± 8.8 211.3–398.4<br>(20) |
| Dog | 133.3 ± 2.3 88.8–220.0<br>(26) | 44.3 ± 1.7 29.5– 67.5<br>(33) | 262.8 ±12.4 159.2–354.2<br>(19) |
| Cat | 77.6 ± 5.9 38.0–119.0<br>(20) | 35.6 ± 4.1 19.0– 87.0<br>(20) | 190.4 ± 7.7 143.5–259.3<br>(20) |
| Rabbit | 44.0 ± 2.6 22.1– 60.0<br>(15) | 43.4 ± 6.8 25.4– 75.0<br>(7) | 221.1 ± 9.2 165.1–294.7<br>(15) |
| Guinea pig | 36.9 ± 2.6 12.0– 62.0<br>(24) | 2.2 ± .4 .4– 7.6<br>(23) | 198.9 ± 6.0 154.9–274.8<br>(25) |
| Hamster | 153.9 ± 8.9 112.0–210.0<br>(12) | | 303.7 ±12.6 208.0–356.0<br>(12) |
| Rat | 85.4 ± 1.9 68.0–103.0<br>(20) | 26.1 ± 1.5 15.6– 39.0<br>(19) | 271.5 ± 6.7 224.1–342.3<br>(20) |
| Mouse | 110.5 ± 8.0 64.0–220.0<br>(20) | | 292.0 ±14.4 225.2–488.4<br>(20) |
| Pigeon | 524.5 ±58.5 325.8–724.5<br>(27) | | 431.3 ±37.8 296.8–570.4<br>(27) |
| Toad *Bufo valliceps* | 152.8 ±26.6 74.8–260.0<br>(10) | | 343.9 ±41.4 208.1–451.8<br>(10) |
| Toad *Bufo fowleri* | 98.1 ±18.8 44.0–238.6<br>(10) | | 237.2 ±17.0 128.4–320.5<br>(10) |
| Frog *Rana catesbeiana* | 69.9 ± 7.8 15.3–136.8<br>(12) | 35.2 ± 3.3 19.3– 61.3<br>(12) | 266.2 ±37.9 169.7–359.7<br>(12) |
| Frog *Rana pipiens* | 28.5 ± 3.3 7.8– 41.2<br>(12) | 28.3 ± 1.4 23.5– 33.9<br>(6)‡ | 395.4 ±22.0 248.7–706.2<br>(24) |

* Mean and its stand. error. † Range of total cholesterol concentrations. No. in parentheses indicates No. of animals used. ‡ Six pooled bile samples from 12 frogs.

liver total cholesterol of the 9 species of laboratory animals studied. The collection of bile from pigeons was not successful because of the extremely small size of the gall bladder. The significance of the high serum and liver total cholesterol of this species is not known. Recently, it was reported that certain breeds of pigeons have a high incidence of spontaneous atheromatous lesions in the aorta that are strikingly similar to those found in man(2).

Total cholesterol concentration in the liver of the amphibian varies with the 4 species studied. These concentrations do not differ greatly from those of the 9 species of laboratory animals. Serum total cholesterol concentrations of both species of toads are higher than those of both species of frogs. Both species of frogs excrete significant amounts of total cholesterol in the bile. Collection of bile from toads was not successful.

*Summary.* 1. Serum, bile, and liver total cholesterol levels of 9 species of laboratory animals and 4 species of amphibians are reported. 2. Dogs, monkeys, hamsters, and mice have relatively higher serum total cholesterol concentration than cats, rats, rabbits, and guinea pigs. 3. Total cholesterol concentration in liver varies only slightly among these species. 4. Gall-bladder bile of monkeys contains large amounts of total cholesterol. Guinea pigs excrete very small amounts of total cholesterol in bile. 5. Serum and liver total cholesterol concentrations of the pigeon are very high. 6. Total cholesterol concentration in liver varies slightly among the 4 species of amphibians used. Frogs excrete significant amounts of cholesterol in bile. 7. Serum total cholesterol concentrations of both species of toads are higher than those of both species of frogs.

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## Plasma Cholesterol Levels in Rats Fed "Infarct-Producing Diets."* (25313)

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Hypercholesterolemia and experimental atherosclerosis have been produced by Wissler, by Fillios and Stare, and others, in rats fed diets containing cholesterol, thiouracil and bile salts(1,2). Only rarely has this atherosclerosis been complicated by coronary thrombosis and myocardial infarction. Recently, Hartroft, Thomas and O'Neal produced thromboses and infarcts in a large percentage of rats fed such diets modified by addition of 40% fat(3,4,5). More recently, Wilgram has also produced myocardial infarcts in rats by a somewhat similar regimen(6). This report presents results of studies of levels of plasma total cholesterol in animals given various modifications of our infarct-producing diet. Details of experiments and anatomic features of the disease have been described (3,4,5).

*Method.* Groups of 10 to 30 rats (Wistar albino males; 80-130 g initial weights) were housed in individual wire-bottom cages in air-conditioned animal room and given water *ad lib.* Semisynthetic diets were prepared according to Table I ("basal ingredients" with partial replacement of sucrose by various fats). Diets were refrigerated and kept no longer than 2 weeks. All rats were offered more food than they would eat, left-overs and scatter weighed, and daily records of food consumption of each animal kept. They were weighed weekly during experiment. Blood specimens were obtained by clipping tails at intervals of 2 weeks, and total cholesterol content in plasma was determined by the method of Pearson, Stern and McGavack(7).

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† U.S.P.H. Trainee in Experimental Pathology.

TABLE I. Characteristics of the Infarct-Producing Diet and Plasma Cholesterol Levels in 5 Experiments.

| No. exp. | Group | Dietary fat and other variations* | No. rats sampled | Plasma cholesterol (mg %) | p values for diff. in values of cholesterol |
|---|---|---|---|---|---|
| I | A | Butter 40% | 11 | 2,570 | <.005 |
| | B | Crisco " | 11 | 1,611 | " |
| | C | Corn oil " | 20 | 717 | " |
| II | D | Butter " | 10 | 1,396 | >.30 |
| | E | Butter-oil " | 16 | 1,327 | >.35 |
| | F | Lard " | 14 | 1,446 | >.30 |
| III | G | Butter " | 11 | 2,570 | <.005 |
| | H | Butter " (no cholesterol) | 30 | 764 | |
| IV | I | Butter " | 34 | 2,248 | " |
| | J | Corn oil 10% (no other fat) | 32 | 1,106 | |
| V | K | Butter 40% | 13 | 1,641 | " |
| | L | Butter " (salt mix 12%) | 6 | 3,315 | |

* Non-fat portions of diet (basal ingredients) which do not appear in the Table but make up remainder of diet consist of cholesterol 5%, propyl-thiouracil 0.3%, sodium cholate 2%, choline chloride 0.2%, salt mix 4%, vitamin mix 2%, Alphacel 6%, casein 20% and sucrose 20.5%, in % by wt of final diet. The salt mix is Wesson modification of Osborne & Mendels—*Science*, 1932, v175, 339. The vitamin mix is Nutritional Biochemicals' "Diet Fortification Mixture" without choline chloride. Changes in the diet noted in parentheses under "other variations" were compensated for by the addition or removal of sucrose in basal ingredients.

After 18 weeks all survivors were killed and autopsied.

*Results.* Plasma cholesterol levels (Table I) represent an average of values obtained between 8 and 18 weeks of the experiment. For rats in Group C, fed basal ingredients plus 40% corn oil, the average of plasma cholesterol levels was 717 mg%, compared with 1,611 mg% in rats of Group B, fed 40% hydrogenated vegetable fat (Crisco), and with 2,570 mg% in those of Group A, fed 40% butter. The difference between any 2 of the 3 groups is highly significant statistically. No significant difference was observed between any 2 of the 3 groups in Exp. II, offered basal ingredients plus 40% butter, 40% butter-oil or 40% lard. Cholesterol levels of rats in Group H, fed basal ingredients without addition of exogenous cholesterol to the 40% butter, averaged 764 mg%, compared with 2,570 mg% for those of Group G, fed regular basal ingredients containing 5% cholesterol plus 40% butter; a highly significant difference. Cholesterol levels of animals in Group J, fed basal ingredients plus 10% corn oil, were 1,106 mg%, significantly lower than the level of 2,248 mg% obtained in Group I, fed basal ingredients plus 40% butter. The amount of salt mix in the diet fed rats of Group L was increased to 12% (with 40% butter) and the average of their cholesterol levels was 3,315 mg%, compared to 1,641 mg% in Group K receiving only 4% of the salt mix with 40% butter.

Fig. 1 is a graph of plasma cholesterol levels of 30 rats of one group fed basal ingredients plus 40% butter during a 4 month experimental period. Levels started to rise within a few days after initiation of the dietary regimen, and reached almost 3,000 mg% at the end of 4 months. Six out of 30 rats in this group developed either single or multiple infarcts of heart or kidneys. The solid line represents levels in rats that developed infarcts, and the dotted line, levels in rats without infarcts. No significant difference was observed between those two ($p = 0.5$).

*Discussion.* Atherosclerosis develops in rats fed low fat diets supplemented with cholesterol (5%), thiouracil (0.3%) and bile salts (2%)(2), but complications of atherosclerosis, such as thromboses and infarcts, are rare. Addition of 40% saturated fat to those diets produces a high incidence of myocardial and renal infarcts(3,4,5). Plasma cholesterol levels of rats fed these infarct-producing diets

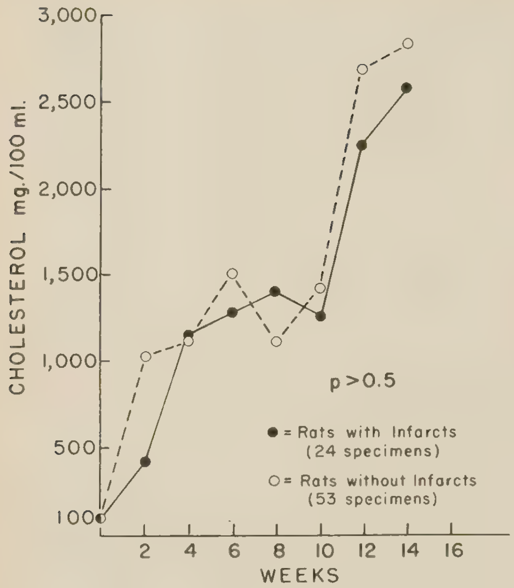

FIG. 1. Plasma cholesterol levels from *one group* of rats with and without infarcts, fed a butter-cholesterolemic diet.

vary from group to group, depending on kind and amount of added fat. It has been established in human and animal experiments that diets containing a large amount of saturated fatty acids tend to cause elevation of blood cholesterol levels(8,9,10). In our studies the basal ingredients plus highly saturated animal fat such as butter or lard, produced the highest plasma cholesterol levels; cholesterol levels were much lower in rats fed basal ingredients plus corn oil, a highly unsaturated fat. The absolute level of plasma cholesterol in the group fed basal ingredients plus 10% corn oil (1,106 mg%) was higher than that of the group fed 40% corn oil (717 mg%), and even those offered only 10% corn oil had significantly lower levels than the comparable group fed 40% butter. Infarcts have not been encountered in rats fed 10% corn oil as sole source of dietary fat except in instances where the dietary supplement of choline chloride was raised from 0.2% to 1.0%. In the latter group, levels of plasma cholesterol were also raised (2,430 mg%) and infarcts occurred(4).

Of interest was the observation of significantly different cholesterol levels between 2 groups of rats given 4% and 12% salt mix in each basal ingredient plus 40% butter. We have no evidence as to the reason for this difference; it may be due to changes in rate of absorption of lipids from the intestinal tract, in transport of absorbed lipids, or in their metabolism.

Of particular interest was the finding that levels of plasma cholesterol in rats that developed infarcts did not rise significantly higher or more rapidly than did those of animals fed the same diet, that failed to develop infarcts. Comparing the various groups of rats, those with higher levels of plasma total cholesterol developed more infarcts than those with lower average levels. But the level of cholesterol at any point in the experiment gave no indication whether an individual rat was or was not more likely to develop an infarct than another rat in the same experimental group. In this regard cholesterol determinations in these experimental models had a somewhat analogous prognostic role to similar determinations in man *i.e.,* they provide a guide to the likelihood of infarction for large groups but not for individuals.

*Summary.* Levels of total cholesterol in plasma of rats fed several variations of a basal infarct-producing diet containing 40% butter, butter-oil or lard were higher than in rats of other groups fed vegetable fat (corn oil or Crisco). Levels of rats in group offered a diet not containing exogenous cholesterol were far lower than those of the control group. A significantly high level was observed when the amount of salt mix in the diet was increased 3-fold to 12%. In a group of 30 rats fed the basal diet, cholesterol in plasma of those that developed infarcts rose neither more rapidly nor to significantly higher levels than did cholesterol in plasma of rats in the same group that did not develop infarcts within experimental period of 4 months.

Author is grateful to Drs. W. Stanley Hartroft, Wilbur A. Thomas and Robert M. O'Neal who kindly allowed me to carry out this study. Their help for interpretation of results and for correction was also invaluable.

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## Metabolism of Radioactive Cholografin in Normal Controls and in Patients with Known Liver Disease. (25314)

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It was shown(1) that concentration of radioactivity by liver following injection of cholografin tagged with iodine 131 was less in dogs with altered liver physiology than in normal dogs. Differences were also noted in clearance rate of radioactivity from blood and excretion of radioactivity in urine. The present study was undertaken to determine whether or not similar results would be obtained with normal controls and patients with known liver diseases.

*Procedure.* Twenty-five μcuries of sodium cholografin‡ tagged with iodine 131 were administered intravenously to normal controls and to patients in whom liver disease had been proven either by needle biopsy or by laboratory studies. The tagged cholografin was diluted to a volume of 1 ml/40 lb body weight with untagged sodium cholografin. This study was also performed on one patient with abdominal enlargement but negative liver function tests and needle biopsy. Individuals were fasted 12 hours preceding the test. Following intravenous injection of tagged cholografin, concentration of radioactivity over liver and bladder was determined by scintillation detectors, ratemeters, and strip chart recorders. Blood samples were drawn at intervals to determine clearance of radioactivity from blood. Two hours after injection of tagged cholografin the subjects were given 5 ounces of water. One hour later urine specimens were collected and percentage of injected radioactivity excreted in these specimens was determined in a deep-well scintillation detector and scaler. Urine and fecal specimens were collected from controls until insignificant radioactivity was present. Urine was collected from patients for 24 hours and feces for 48 hours. The percentage of original radioactivity excreted in urine and feces was calculated for these periods. Controls were 4 young healthy adults with no history or signs of liver disease and on whom serum bilirubin levels were normal.

*Results.* In normal subjects the level of radioactivity over the liver increased at least 30% above equilibrium level recorded immediately after complete mixing of injected cholografin in blood volume. In patients with proven liver disease there was no concentration of cholografin by the liver and in several cases there was a decrease of 60% of radioactivity over the liver in 30 minutes. Representative curves are illustrated in Fig. 1.

Concentration of radioactivity in bladders of normal subjects increased slightly over a 30 minute interval with a maximum increment of approximately 15%. In a similar period of time 3 of 5 patients with proven liver

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‡ Supplied by Squibb Inst., New Brunswick.

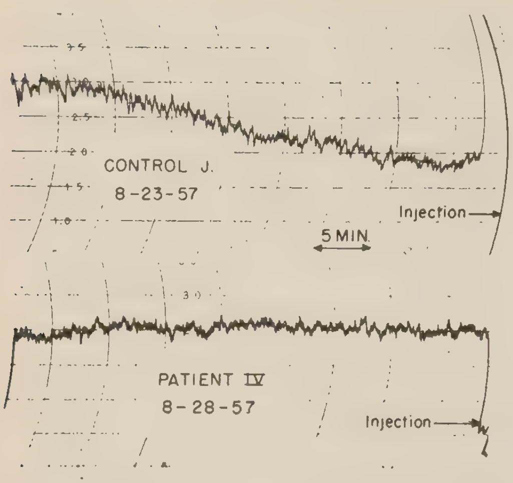

FIG. 1. Representative curves of concentration over the livers of one of the controls and one of the patients.

disease had increases in radioactivity of 40, 70, and 55% and the other 2 patients had an increase of 12%.

The time necessary to clear one-half the radioactivity from blood stream in controls varied from 80 to 150 minutes, averaging 112 minutes. In 4 patients with liver disease the time was in excess of 210 minutes; in the fifth case, 160 minutes.

The percent radioactivity in 3 hour urine specimens of controls ranged from 4.6% to 7.4%. The average was 5.9% with a standard deviation of 1.1%. The 24 hour urine specimens varied from 14.3 to 17.2% with average of 15.6% and standard deviation of 1.2%. The 3 hour urine specimens on patients with liver disease ranged from 6.1% to 19%. Two cases were within the range of normals. On 24 hour urine samples the range was 16.1 to 36%. The lowest of these was on one patient with a normal 3 hour excretion and was the only one within the normal range for 24 hours excretion.

Total radioactivity collected in feces of controls ranged from 52 to 75%. After 48 hours very little radioactivity was recovered (less than 10%). In patients with liver disease recovery of radioactivity in stool specimens for 48 hours varied from 0.45 to 11%. In one patient with obstructive jaundice there was no radioactivity in the 24 hour specimen. The patient was taken to surgery before a 48 hour specimen could be obtained.

On the patient with psychosomatic complaint of abdominal enlargement and normal liver function tests and liver biopsy the above tests were all similar to those found in the normal subjects. The results of controls are shown in Table I and those of patients with liver disease in Table II.

*Discussion.* The differences in results between control and patient groups closely parallel earlier results of animal work.

TABLE I. Values in Control Group.

| Test | Controls | | | | | |
|---|---|---|---|---|---|---|
| | T | J | C | B | $\bar{X}$ | s |
| $T\frac{C^*}{2}$ | 150 | 80 | 100 | 120 | 112 | 30 |
| $\frac{T\ 2\text{ hr}\ \dagger}{T\ 1/2\text{ hr}}$ | .60 | .42 | .43 | .55 | .50 | .08 |
| 3 hr urine (%) | 5.5 | 5.9 | 7.4 | 4.6 | 5.9 | 1.1 |
| 24 ” ” (%) | 15.6 | 17.2 | 15.8 | 14.3 | 15.6 | 1.2 |
| Fecal recovery (%)‡ | 67 | 72.0 | 52.7 | 52.0 | | |
| Inc. activity§ over liver (%) | 30 | 50 | 88 | 40 | | |
| ” ” § ” bladder (%) | 15 | Slight | Slight | 15 | | |

* $T\frac{C}{2}$ = Time (min.) needed for radioactivity in blood to be reduced to ½ the value immediately following inj.

† $\frac{T\ 2\text{ hr}}{T\ 1/2\text{ hr}}$ = Ratio of radioactivity in the blood 2 hr after inj. of cholografin to radioactivity in blood ½ hr after inj.

‡ For 3-4 day period. After 48 hr recovery was less than 10%.

§ Areas monitored for 30 min. following inj. of radioactive cholografin.

TABLE II. Values of Liver Function Tests in Patients.

| Patient<br>Diagnosis | I<br>CA pancreas | II<br>Inf. hepatitis | III<br>Cirrhos. | IV<br>Cirrhos. | V<br>Cirrhos. | VI<br>Psychosomatic | Normal values |
|---|---|---|---|---|---|---|---|
| BSP, 60 min. (%) | | | 20 | 30 | Neg. | <5 | <5 |
| Thymol turbidity | 5.5 | 15.4 | | 17.5 | | 3.7 | .4–4 |
| Alk. phosphatase (Bodansky units) | 5.4 | 5.2 | | 2 | 3.05 | 1.2 | 1–4 |
| Bilirubin (mg) | 22.5 | 18.2 | 9.8 | 1.75 | 2.40 | .75 | .2–1 |
| Total protein | 7.0 | 6.8 | 5.8 | 8.3 | 6.1 | 7.9 | 6–8 |
| Albumin | 3.3 | 3.0 | 2.5 | 2.2 | | 4.7 | 2–4 |
| Globulin | 3.7 | 3.8 | 3.3 | 6.1 | | 3.2 | 1–2 |
| Prothrombin (%) | 90 | 100 | 60 | 45 | | 100 | 70–100 |
| Thymol floc. | | 2+ | | 3+ | | 0 | <1 |
| Bile in urine | | 4+ | 0 | 0 | | 0 | 0 |
| Cholografin studies | | | | | | | |
| Inc. activ., liver (%) | − 5 | − 60 | − 8 | 0 | 0 | +60 | >+30 |
| " " , bladder (%) | +40 | +70 | +12 | +55 | +12 | +12 | < 15 |
| T C/2 (min.) | >210 | >210 | >210 | 160 | >210 | 135 | 112 ± 30 |
| T 2 hr/T ½ hr | .81 | .83 | .81 | .65 | .81 | .63 | .50 ± .08 |
| 3 hr urine (%) | 10.6 | 7.3 | 6.1 | 19 | 6.7 | 4.4 | 5.9 ± 1.1 |
| 24 " " (%) | 20.6 | 43.3 | 16.1 | 27.4 | 18.1 | | 15.6 ± 1.2 |
| 48 " fecal (%) | 0 (24 hr) | .46 | 11.0 | 9.8 | 3.6 | 64.0 | >42 |

Of various tests performed, the sharp contrast in build-up of radioactivity over the liver between controls and patients with liver disease is the most significant and most consistent. Lack of significant concentration of cholografin by the diseased liver affords a simple method of evaluating liver function.

Determining percentage of injected radioactivity excreted in urine is no more difficult than monitoring the liver, but several patients in our small series failed to excrete in excess of the normal range. Nevertheless, the differences between means of the 2 groups are statistically significant. If this procedure is used, the added parameter of renal function must be considered. Probably correlation of specific gravity of morning urine specimen would suffice.

The differences between the 2 groups in clearance of radioactivity from blood and in excretion of radioactivity into the bowel were also significant, but not as striking as in monitoring the liver. These tests are also somewhat objectionable because of the necessity of multiple venepunctures for the former and the difficulty and unpleasantness of collecting stool specimens for the latter.

The time necessary for radioactivity of blood to decrease to one-half its original value and the ratio of radioactivity of blood at 2 hours to that at one-half hour after injection are a function of the same process. The latter procedure requires only 2 venepunctures, but the accuracy derived from multiple determinations of radioactivity is lost.

Before deciding on a routine dose of 1/40 ml of cholografin/lb of body weight, various determinations were performed on controls using .15 ml of cholografin/lb of body weight and undiluted tagged cholografin (less than .0075 ml/lb). The results were similar to these presented here, except that urinary excretion was slightly higher when greater total doses of cholografin were administered. Since 1/40 ml/lb of body weight permitted use of a supply of tagged cholografin even after it decayed to a specific activity of approximately 6 $\mu$c/ml and since this dose did not produce nausea in any of the subjects, it was subsequently used.

*Conclusions.* 1. Hepatic uptake, clearance of radioactivity from blood, and excretion of radioactivity into urine and feces of patients with liver disease differ signficantly from similar determinations in controls.

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# Metabolism of Thyroxine in Larva of Rice Moth (*Corcyra cephalonica* St.)

(25315)

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Our earlier investigations(1,2) have clearly established that the insect, *Corcyra cephalonica* St., responds to administration of thyroxine in a manner strikingly similar to that observed in mammals. This similarity could be extended to alterations in the catalase and amylase levels as well as to existence of a direct thyroxine-Vit. $B_{12}$ antagonism(3). The greater effectiveness of triiodothyronine in the insect as well as in mammals raises the question whether thyroxine might be deiodinated to triiodothyronine before exerting its action. *In vitro* conversion of thyroxine to triiodothyronine occurs in mammalian tissues(4,5,6). The results of our study of *in vitro* and *in vivo* metabolism of thyroxine in *Corcyra* are here presented. Excretory metabolism of thyroxine in *Corcyra* studied by chromatographic methods, showed iodide to be the major metabolite. *In vitro* experiments with homogenates of *Corcyra* larvae established that deiodination of thyroxine was not a normal feature of insect metabolism, but occurred only in larvae reared on thyroxine-supplemented diet.

*Materials and methods.* Chromatographically pure radioactive l-thyroxine (labeled in the 3′ and 5′ positions) was obtained from Abbott Labs, Chicago, Ill., and had a specific activity of 29 μc/μg. The stock solution in 50% propylene glycol was diluted with water to the desired concentration. Cell-free extracts of tissues of larvae reared on control diet of wheat flour and on diets supplemented with thyroxine (2 μg/g of diet) were tested for activity to deiodinate thyroxine. Larvae maintained on control and thyroxine-supplemented diets for 20-25 days were picked and allowed to starve for about 3 hours. After cleaning thoroughly with a camel hair brush, they were ground in previously chilled mortar and homogenized in ice-cold isotonic potassium chloride in Potter-Elvehjem glass homogenizer equipped with Teflon pestle (Arthur H. Thomas and Co.). The homogenate was filtered through cotton wool and used as the enzyme source. *Analysis of larval excreta. Corcyra* larvae were reared on thyroxine-supplemented diets(2). Thyroxine was included at concentration of 2 μg/g of diet. After most of diet had been consumed, fecal pellets were separated from diet particles and cleaned well with camel hair brush. The excreta (500 mg) were then extracted twice with 5 ml portions of ethanol-2N ammonia (1:1) mixture. After centrifuging, the supernatant was concentrated *in vacuo*. The residue was dissolved in suitable volume of ethanol ammonia mixture and aliquots were taken up for chromatography. *Paper chromatography* was carried out on Whatman No. 1 filter paper. In each experiment, at least 2 of the following systems were employed. 1. n-butyl alcohol: acetic acid: water 78:10:12 or 78:5:17. 2. *idem* : dioxane : 2N ammonia 4:1:5. 3. *idem* ethyl alcohol : *idem* 5:1:2. In chromatographic experiments with radioactive materials, the samples were usually supplemented with suitable amounts of carrier substances. The positions of these substances were located by application of the ceric sulphate-arsenious acid reagent of Bowden *et al.*(7) as modified by Dragunova and Langer(8). Radioautographs of the chromatograms were made on Ilford-Ilfex X-ray films. All radioactive measurements were made in a well type scintillation detector (DS-S-5) attached to precision rate-meter (Nuclear Chicago Corp.). Measurements were corrected for background and decay. Distribution of radioactivity on chromatogram strips was determined by cutting each strip into segments of 0.5 cm width and directly counting individual segments. The activity corresponding to each spot was expressed as percent of total activity in the strip. The incubation medium for examination of deiodinase activity of larval homogenates comprised 2 ml of appropriate enzyme solution, 0.1 ml of substrate solution and buffer to make up the volume to 3 ml. Blank and control tubes received respectively 2 ml of isotonic KCl and boiled (10-15 minutes) larval homogenates in place of the enzyme solution. Incubations were carried out at 37°C for 5 or 16 hours with occasional shaking. *Analysis of incubation media.* Inorganic iodide, which was the major radioactive metabolite, was estimated as follows: 0.5 ml aliquots of various incubation mixtures were withdrawn into tubes containing 1 ml of 20% trichloroacetic acid (TCA). The contents were filtered, the precipitate washed twice with 0.5 ml of 5% TCA solution, and filtrate and washings pooled. TCA was then removed by repeated extraction with ether until pH came down to about 3-4. Ether extract was washed once with dilute thiosulphate solution and the washings added to original solution. 100 mg of deactivated charcoal(9) was then added to the solution (pH 3-4) and the tube shaken vigorously. At the end of 30 minutes, the contents were centrifuged, and the supernatant transferred carefully to another tube and radioactivity measured. This represents the inorganic iodide fraction. In experiments where a search for other possible metabolites was made, chromatographic analysis was carried out to get both quantitative and qualitative assessment of the compounds. Further, the method served as a check on the value of inorganic iodide fraction as obtained by procedure outlined above.

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*Results. Nature of excretory metabolites of thyroxine.* Chromatographic analysis of excreta of larvae fed thyroxine and comparison with authentic samples established the presence of thyroxine and iodide in the excreta. Control experiments carried out with excreta of larvae fed control diet of wheat flour alone, showed no distinct iodine-containing compounds. The fact that iodide was the major identifiable metabolite suggested the presence of a deiodinating mechanism in the larvae. Further, the chromatograms often showed faint spots corresponding to monoiodotyrosine and diiodotyrosine, thus suggesting that a fission at the ether linkage is another aspect of metabolism of thyroxine in the larvae.

*In vitro deiodination of thyroxine by larval homogenates.* 10% (w/v) homogenates of larval tissues were employed. Thyroxine was included in mixtures at concentrations ranging from $10^{-6}$ M to $10^{-8}$ M. The average results of replicate experiments presented in Table I demonstrate conclusively that tissues of thyroxine-fed larvae are capable of forming iodide from thyroxine, whereas, larvae reared on normal diets lack this ability. In many replicate experiments the extent of deiodination was about 10%, under conditions employed. Deiodination of thyroxine was studied at pH values of 7.2 and 8.6 (phosphate buffer) and 9.5 (carbonate-phosphate buffer). The results presented in Table I indicate that the enzymic deiodination of thyroxine occurred over a wide range of pH.

*Chromatographic analysis of the incubation media.* Chromatographic analysis of both control and experimental incubation media of

TABLE I. Deiodination of Thyroxine by Larval Homogenates. The test system contained 2 ml of larval homogenate, 0.1 ml of radioactive thyroxine (conc. $1.24 \times 10^{-7}$ M and activity $1.5 \times 10^5$ cpm) and 0.9 ml of appropriate buffer solution. Incubation period 5 hr at 37°C. Iodide was determined by charcoal treatment procedure as described in text.

| | | % iodide formed* at pH value of | | |
|---|---|---|---|---|
| Nature of homogenate | Exp. system | 7.4 | 8.6 | 9.3 |
| Tissue of larvae fed control diet | Blank | 2.4 ( 2.0– 2.7)† | | |
| | Boiled enzyme | 2.5 ( 2.1– 2.7) | | |
| | Test | 2.7 ( 2.6– 2.9) | | |
| Tissue of larvae fed diet containing thyroxine | Blank | 2.9 ( 2.6– 3.2) | 2.6 ( 2.3– 2.8) | 1.9 ( 1.4– 2.3) |
| | Boiled enzyme | 5.9 ( 5.1– 6.4) | 5.4 ( 5.1– 5.6) | 5.8 ( 5.3– 6.2) |
| | Test | 14.8 (13.7–16.0) | 15.3 (14.8–16.0) | 17.0 (16.6–17.3) |

* Results are expressed as $\frac{\text{Radioactivity in inorganic iodide fraction} \times 100}{\text{Total activity added to incubation medium}}$.

† Values in parentheses represent range.

homogenates of larvae fed control diet revealed feeble activity (less than 2%) in areas corresponding to iodide. These results confirm the absence of deiodinase system in control larvae. The results of a typical experiment with the homogenates of thyroxine-fed larvae at pH 8.6 are represented in Fig. 1. In the control as well as blank, little or no deiodinase activity was observed. The extent of deiodination in the experimental averaged from the replicate experiments was 10.9%. These results are in close agreement qualitatively and quantitatively with those obtained by the charcoal treatment procedure.

Besides thyroxine and iodide, another radioactive area having an $R_f$ of 0.5 in both butanol : acetic acid : water and butanol : dioxane : ammonia solvent systems, was noticed. This spot accounted for less than 10% of total activity of the chromatogram and further, the activity of this spot was comparatively greater in chromatograms of short-term incubation mixtures. Detailed analysis of time-course curves of the degradation of thyroxine might reveal the significance of this factor in deiodination of thyroxine.

*Discussion.* The possibility of iodide being formed in *in vitro* experiments as a consequence of chemical interaction between thyroxine and some compounds in tissues, has been stressed by Michel(10). Such a possibility could however be eliminated since no iodide could be detected in boiled enzyme blank either by charcoal adsorption procedure (Table I) or by chromatographic method (Fig. 1). The consistent presence of iodide in chromatograms developed with acidic or alkaline solvents further establishes that it is not an artifact formed during chromatography.

Two-dimensional chromatography of the incubation mixtures failed to reveal formation of triiodothyronine in most experiments. However, as experienced by Tata *et al.*(11), a spot corresponding to triiodothyronine also showed up occasionally in our experiments. Thus, presence of triiodothyronine in the incubation media was not a consistent feature. In the light of these observations, it is difficult to judge the importance of the deiodinating mechanism in conferring biological activity to thyroxine molecule.

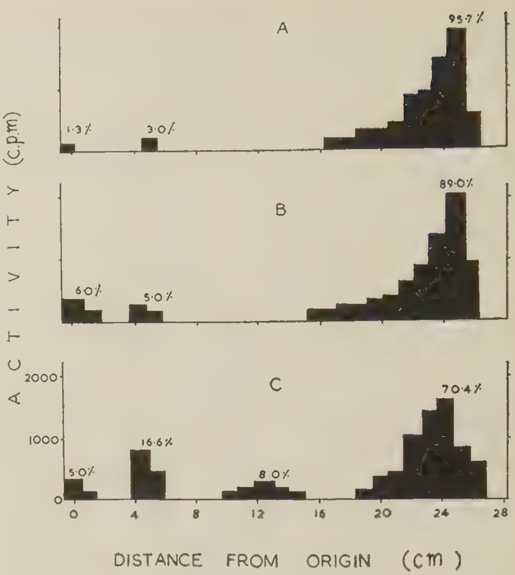

FIG. 1. Deiodination of $I^{181}$-labeled thyroxine by 10% homogenate of thyroxine-fed larva. Distribution of radioactivity in chromatogram. Substrate conc. $2.48 \times 10^{-7}$ M; incubation period 5 hr at 37°C in phosphate buffer pH 8.6; solvent system—butanol : acetic acid : water, 78 : 5 : 17. Values expressed as % of total activity in the entire chromatogram. A—Blank; B—Boiled enzyme control; C—Test. Shaded areas in C correspond to origin, iodide, unknown, and thyroxine.

Attempts to detect acetic or propionic acid analogs of thyroxine in the incubation mixtures led to results which were not unequivocal, inasmuch as authentic samples of these compounds added to the larval homogenates tended to streak along or coincide with thyroxine spot on the chromatograms.

The observation that enzyme activity is exhibited only by larvae fed thyroxine and not by larvae grown normally, suggests the possibility that the enzyme is elaborated only under stress imposed by thyroxine, presumably as a detoxication mechanism. Thus, synthesis of the enzyme may represent an adaptive response on the part of the insect organism to the thyroxine. Further work in progress, on purification of the enzyme as well as a study of enzyme levels at different periods of thyroxine feeding, might throw more light on this interesting phenomenon.

*Summary.* The major excretory metabolite of thyroxine in the insect *Corcyra cephalonica*

St. is iodide, though monoiodotyrosine and diiodotyrosine are present in small amounts. *In vitro* experiments with homogenates of larval tissue have shown that an enzyme system capable of forming iodide from thyroxine is present only in tissues of larvae fed thyroxine-supplemented diets and not in those of larvae fed control diet alone. Activity of the enzyme is destroyed by boiling for 10-15 minutes.

The authors are grateful to Abbott Labs., Chicago, for a generous gift of radioactive l-thyroxine. One of the authors (P.V.N.) acknowledges the award of a Research Training Scholarship by the Government of India.

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## Isolation and Identification of Testosterone in Systemic Blood from Normal Human Male Adults.*† (25316)

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It is well recognized that testosterone and $\Delta^4$-androstene-3,17-dione are the predominant steroid hormones of the human testis(1,2). These androgens have also been found in canine spermatic vein blood(3) and in dogs, spermatic vein concentration of testosterone and $\Delta^4$-androstene-3,17-dione increases markedly after human chorionic gonadotropin (HCG) administration(4). Heretofore, neither testosterone nor $\Delta^4$-androstene-3,17-dione have been reported in systemic blood of man. In the present communication, isolation of testosterone from systemic human blood after administration of HCG is described.

*Materials and methods.* Chemicals, unless otherwise specified, were reagent grade and all solvents used were freshly distilled. Evaporation was done at +40°C under nitrogen. Blood was obtained from 3 normal human male subjects 2 hours after intramuscular injection of 1000 units HCG.‡ Heparin was used as anticoagulant and a plasma pool of 400 ml was processed. The plasma was extracted 4 times with 500 ml ethylacetate/ether (1:1 v/v) each time. The combined extracts were evaporated to dryness and the residue suspended in 25 ml 70% methanol (MeOH) and stored at −15°C for 15 hours. The MeOH solution was then centrifuged 15 minutes at 2000 rpm in a refrigerated centrifuge. The supernatant was carefully removed, the precipitate consisting of lipid material(5) was washed once with 5 ml ice cold 70% MeOH, and the MeOH extracts were combined. Last traces of lipids were removed from the combined 70% MeOH solution by extraction with 30 ml n-hexane. The hexane was discarded and the MeOH solution was evaporated to drynes. The dry residue was transferred to a paper strip with chloroform-MeOH (1:1 v/v) and on another strip 50 γ synthetic testosterone were applied in the same fashion (6). Ascending chromatography was then performed with MeOH-benzene (1:9 v/v) to concentrate the material at tip of strip. The tips of these concentration strips were cut off, inserted into appropriate cuts on strips of formamide-MeOH impregnated paper and subjected to descending chromatography in formamide-benzene(7). The chromatograms were removed from the tank and carefully dried when the solvent front reached the lower end of strip. The Rf value of standard testosterone was determined by a Haines UV scanner and the area of the sample strip corresponding to the testosterone spot on the standard strip was eluted with MeOH and evaporated to dryness. The dry residue was rechromatographed in propylene glycol-methylcyclohexane(8) using 50 $\gamma$ desoxycorticosterone (DOC) as reference compound. Relative mobility of testosterone compared to DOC was 0.68-0.72. This area of the sample strip was eluted and the dried eluate subjected to chromatography on 1.5 g purified silica gel(9), prepared wet with 0.5% ethanol (EtOH) in methylene chloride ($MetCl_2$). Unknown material was applied in 0.5% EtOH in $MetCl_2$. The column was then washed with 25 ml of 1% EtOH in $MetCl_2$ and the fraction 5% EtOH in $MetCl_2$ collected, since authentic testosterone was eluted from silica gel in this fraction. A blank column was processed simultaneously. The final fraction from the column containing the plasma sample was evaporated to dryness and the residue dissolved in 1 ml MeOH. The ultraviolet absorption spectrum of the sample was recorded in a Beckman DK 2 spectrophotometer using 5% EtOH in $MetCl_2$ fraction from the blank column as reference. One-tenth of unknown sample was then evaporated and acetylated with $C^{14}$ labeled acetic anhydride(10), while the rest was submitted to infrared analysis in a Perkin Elmer Model 21 infrared spectrophotometer with ultramicro sampling system (11).[§] The acetylated sample was chromatographed in propylene glycol-methylcyclohexane(8) and the radioactivity on the paper was localized by scanning in a strip counter. The area of the chromatogram containing radioactivity was eluted and the eluate divided. To one part was added 25 $\mu$g authentic testosterone acetate. The sample was chromatographed in formamide-hexane(12). Localization of radioactivity on the strip was determined in a strip counter and scanning of the strip at 240 m$\mu$ was subsequently done by UV scanning devise attached to a Beckman DU spectrophotometer. The second part was dissolved in 15 ml ethyl acetate and washed with 10 ml of a solution consisting of 0.2% anisaldehyde in 0.3 ml absolute EtOH, 0.7 ml conc. sulfuric acid, made up to 100 ml with distilled water. The ethyl acetate was thereafter washed twice with 10 ml water, evaporated to dryness and the residue submitted to a color reaction giving a pink color with peak absorbance at 510 m$\mu$ for testosterone, testosterone acetate and $\Delta^4$-androstene-3,17-dione. In this reaction the steroid is dissolved in 0.15 ml of 0.2% anisaldehyde in absolute EtOH, 0.35 ml conc. sulfuric acid is added, the sample is placed in boiling water bath (+92°C) for 10 minutes, cooled under tap water and transferred to a micro cuvette of 0.5 ml capacity. Absorbance is determined in a Beckman DU spectrophotometer at 450, 510 and 570 m$\mu$. The color produced is stable for at least 3 hours at +23°C. If the Allen correction(13), modified by Brown(14) is used to correct maximum absorbance at 510 m$\mu$, 1 $\gamma$ testosterone dissolved in 1 ml of the described reagent gives a mean absorbance at this wavelength of 0.183 + 0.011[‖] (n = 25).

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† The authors express their appreciation to Drs. Leo T. Samuels and Richard Nimer.

‡ Obtained as a gift from Upjohn Co., Kalamazoo, Mich.

*Results.* The material in plasma behaving like testosterone in 2 paper chromatographic systems exhibited a distinct UV absorption maximum at 241 m$\mu$. A total of 5.6 $\gamma$ testosterone was present in 400 ml of plasma. The infrared absorption spectrum strongly indicates that this material is testosterone, though minor additional absorption bands were observed, probably due to admixture of contaminating material (Fig. 1). However, in the fingerprint region adequate infrared resolution was present showing spectral agreement between authentic testosterone and the material isolated from plasma.

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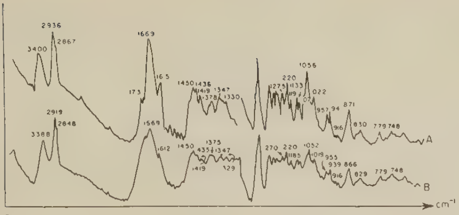
[§] We are indebted to Dr. Dale A. Clark, Veterans Hosp., McKinney, Texas for the infrared analysis.

[‖] Stand. dev.



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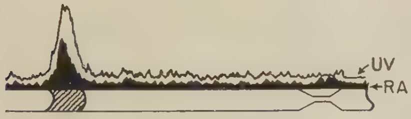

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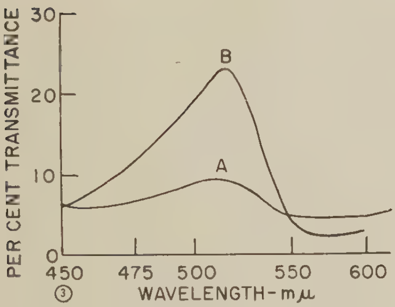

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FIG. 1. Infrared spectra of testosterone (A) and "testosterone" from plasma (B).

FIG. 2. Tracings from UV and radioactive scanning: A mixture of testosterone acetate and $C^{14}$ acetate of plasma "testosterone" was used. RA: radioactivity. UV: UV absorbance at 240 mμ. The marked area on chromatogram represents UV absorbance as detected by Haines scanner.

FIG. 3. Spectral curves of testosterone acetate subjected to anisaldehyde-ethanol-sulfuric acid. A: acetylated unknown material in plasma approximately 0.3 γ in 0.7 ml reagent. B: authentic testosterone acetate, 1.5 γ in 0.7 ml reagent.

The compound from plasma formed an acetate with acetic anhydride-$C^{14}$ which behaved chromatographically like testosterone acetate. When the $C^{14}$ labeled acetate of the compound was diluted with non-labeled authentic testosterone acetate and chromatographed in formamide-hexane, the peak of radioactivity coincided with that of the UV absorption maximum at 240 mμ (Fig. 2). Furthermore, the $C^{14}$ labeled acetate of the compound reacted with the anisaldehyde-EtOH-sulfuric acid to give an absorption maximum at 510 mμ like authentic testosterone acetate (Fig. 3).

One hundred and fifty ml pooled plasma from normal male subjects not given HCG were assayed for testosterone by methods described. In this plasma pool, no testosterone was found.

*Discussion.* The data indicate that testosterone actually is present in the systemic circulation of normal male adults after administration of HCG. A concentration of testosterone 2 hours after injection of human chorionic gonadotropin was approximately 1.4 γ/100 ml plasma. Gallagher *et al.*(15) isolated deuterium-labeled androsterone following administration of deuterium-labeled testosterone in man, and we recently demonstrated that intramuscular administration of HCG increases conjugated plasma androsterone in the normal male(16). This increase is not seen in the normal woman. With the actual identification of testosterone in male plasma at the time when the metabolite is increased in the blood, it can be concluded that human chorionic gonadotropin promotes a prompt secretion of testosterone in normal human male similar to that already demonstrated in the normal male dog(4).

*Summary.* By criteria of paper chromatography in 2 systems, formation of an acetate derivate, ultraviolet and infrared spectrophotometry, testosterone has been isolated and identified in systemic blood of normal male subjects given HCG intramuscularly. Plasma testosterone could not be found in normal male subjects not stimulated with HCG.

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# Hemagglutination of Rabbit Erythrocytes by Pus from Cases of Cat Scratch Fever. (25317)

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The possible viral etiology of cat scratch fever, non-bacterial regional lymphadenitis, has been postulated for some time. The demonstration of positive skin tests and complement-fixing antibodies to lygranum in patients with this disease, as well as observation of cytoplasmic, basophilic inclusion bodies similar to those seen in psittacosis, have indicated specifically a virus related to the psittacosis-lymphogranuloma venereum group. Although Mollaret(1) has been able to transmit the disease in humans and monkeys using filtrates of pus from lymph nodes, numerous efforts to isolate an infectious agent, by use of conventional laboratory media, inoculation of common experimental laboratory animals, and use of tissue culture explants, have failed. This paper is a preliminary report on hemagglutination of rabbit red cells by pus aspirated from lymph nodes of clinical cases of cat scratch fever as possibly a viral type of hemagglutination, as well as attempts to demonstrate specific hemagglutinin inhibiting antibody in serum of patients with this disease, and in rabbits immunized with pus.

*Materials and methods* for hemagglutination of rabbit red cells consisted of pus from lymph nodes of patients clinically diagnosed as cat scratch fever on the basis of a regional non-bacterial lymphadenitis and a history of one or more cat scratches. The pus, as received, was diluted 1:5 in physiological salt solution and 2 of the samples had been heated at 56°C for one hour on consecutive days for preparation of skin-test antigen. This material, either untreated or heated, was serially diluted in phosphate buffered saline through 1:512. One drop of each dilution was then mixed with one drop of a 2% suspension of washed rabbit red cells. The tubes were incubated in water bath at 37°C for 30 minutes, after which they were centrifuged lightly at 1500 rpm for 30 seconds, and observed for hemagglutination by shaking the tubes gently and noting appearance of clumped red cells. Trypsinized red cells were prepared by treating the suspensions with 0.1% trypsin (Difco) for 30 minutes in water bath at 37°C. The preparation of tanned cells consisted of similar treatment with dilute tannic acid. The human sera employed consisted of samples from 2 infected individuals from whom pus was obtained; serum from a person known to have a positive skin test; and serum from an individual suffering from infection with adenovirus. The rabbit sera were obtained before and after immunization of the animal with his own red cells previously treated with pus. Tests for hemagglutinin inhibiting antibody consisted of incubating equal volumes of various dilutions of serum and a dilution of pus containing 4 hemagglutinating units for 30 minutes in water bath at 37°C and then testing for hemagglutination with red cells as described above.

*Results.* Rabbit erythrocytes were agglutinated by all 10 samples of pus from known

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TABLE I. Titration of Hemagglutinin in 10 Pus Samples.

| | | Reciprocal of dilutions | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Sample No. | Skin test | 2 | 4 | 8 | 16 | 32 | 64 | 128 | 256 | 512 |
| 1502 | Not known | S | S | S | S | 4+ | — | — | — | — |
| 1550 | Not done | S | S | S | S | 2+ | — | — | — | — |
| 1263 | + | S | S | 4 | 3+ | — | — | — | — | — |
| 11-28-1 | + | S | S | S | S | 4+ | — | — | — | — |
| -2 | + | S | S | 2+ | 2+ | — | — | — | — | — |
| -3 | + | S | S | 4+ | 4+ | — | — | — | — | — |
| -4 | + | S | S | S | S | 2+ | — | — | — | — |
| -5 | + | S | 4+ | — | — | — | — | — | — | — |
| AK-1 | — | 4+ | 4+ | 4+ | 4+ | 4+ | 4+ | 4+ | — | — |
| XE-1 | — | 3+ | 2+ | 2+ | 2+ | 2+ | + | + | + | — |

S = All cells agglutinated in a single, large clump.

or suspected cases of cat scratch fever including the 2 from skin test negative cases (Table I). Although titers of the material from these latter 2 cases were higher, amount of agglutination in all tubes was not equal to maximal agglutination obtained with samples from skin test positive cases. Samples #1502 and #1550 had been heated to 56°C previous to testing. The same results were obtained with 3 samples using trypsinized and tanned red cells, but human and sheep cells were not agglutinated. Similar tests were negative with purulent lymph node material from 3 cases of lymphadenitis having no clinical or skin test evidence of cat scratch fever.

Table II shows results of tests performed on human and rabbit sera for presence of hemagglutinin inhibiting antibody employing pus from case #1263 diluted so as to contain 4 hemagglutinating units. Only one of the 4 human sera tested (#1263) and the immune rabbit sera demonstrated any antibody.

To confirm specificity of the inhibition, aliquots of serum #1263 and immune rabbit serum were incubated with diluted pus (#1263) and with hemagglutinin negative purulent fluid from a case of lymphadenitis clinically unlike cat scratch fever. The sera were then diluted and retested for antibody with 4 hemagglutinating units of pus as previously described. Inhibiting antibody was absorbed from both sera only by pus from cat scratch fever.

To elute a presumed virus, rabbit cells sensitized with pus were resuspended in saline and incubated at 10°C, 25°C and 37°C for 4 hours. Tests for hemagglutinin in eluates obtained by centrifugation using fresh rabbit cells were negative.

*Discussion.* Lack of previous evidence of hemagglutination in cat scratch fever and the possibility that the procedures described may be useful in diagnosis of the disease prompted this preliminary report. Good evidence that the hemagglutinin is a virus has been obtained in our laboratory and will be published.

*Summary.* Hemagglutination of rabbit red cells with pus obtained from lymph nodes of 10 cases of cat scratch fever was demonstrated.

TABLE II. Titration of Hemagglutinin Inhibiting Antibody in Human and Rabbit Sera.

| | | | Reciprocal of serum dilutions | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Serum | Clinical diagnosis | Skin test | 2 | 4 | 8 | 16 | 32 | 64 | 128 | 256 | 512 | 1024 | 2048 |
| 1502 | CSF | Not known | S | S | S | S | S | S | S | S | S | S | S |
| 1503 | Adenovirus | Neg | S | S | S | S | S | S | S | S | S | S | S |
| 1550 | CSF | Not done | S | S | S | S | S | S | S | S | S | S | S |
| 1263 | CSF | Pos | — | — | — | — | — | — | S | S | S | S | S |
| Normal rabbit | — | — | S | S | S | S | S | S | S | S | S | S | S |
| Immune rabbit | — | — | — | — | — | — | — | — | — | — | 3 | 3 | 3 |

S = All cells agglutinated in single, large clump. CSF = Cat scratch fever.

Hemagglutinin control—#1263 pus diluted in saline to contain 4 hemagglutinating units—"S" agglutination.

Specificity of hemagglutinin was indicated by inhibition by serum from a known case of the disease and by rabbit antiserum to the pus.

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## Accumulation of *para*-Aminohippurate by Slices of Kidney from Rabbits of Various Ages.* (25318)

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Previous investigations have shown a gradual increase in renal tubular function *in vivo* from fetal life to maturity. Williamson and Hiatt(1) observed rapid increase of phenolsulphonphthalein (PSP) excretion by rabbit kidney from 26th day of gestation to 10th day postnatally when PSP excretion attained adult levels. In the rat(2) ability of tubules to absorb an intravital dye such as trypan blue began at 12 days of age and reached a maximum at 28 days; intravital staining was directly correlated with acquisition of a brush border. Levine and Levine(3) working with fetal, newborn and adult rabbit also report a gradual increase in $C_{PSP}$ until 1 month of age. Experiments on development of renal tubular function *in vitro* cover only the post-natal period. Deyrup(4) demonstrated a low $S^{35}$ uptake in the newborn rat when compared to adult. Rennick(5) observed that transport systems for tetraethylammonium (TEA) and *para*-aminohippurate (PAH) developed at different ages in the puppy: that for PAH at 3-4 weeks of age and that for TEA at 7 weeks of age. This report extends investigation of development of renal tubular function *in vitro* into the fetal period. Accumulation of PAH and chlorphenol red (CPR) by kidney slices is used as a measure of tubular function from fetal life to maturity in the rabbit. Contrary to previous reports(1-5) these data show that instead of a gradual increase in function with age there is less PAH accumulation in the slice from newborn than from either fetus or adult.

*Methods.* Rabbits of various ages were used: fetuses of 26 to 28 days gestational age; nurslings from 6 hours to 22 days; adult males and females, pregnant and non-pregnant. Essentially methods described by Cross and Taggart(6) were utilized throughout. Slices weighing 200-300 mg(7) were incubated at 30°C for 60 minutes in Krebs-Ringer phosphate(8) with either PAH or chlorphenol red $7.4 \times 10^{-5}$ M. Control flasks containing $5 \times 10^{-5}$ M dinitrophenol were included in most experiments. At end of incubation, slices were removed, blotted and placed in 10% trichloroacetic acid for PAH determination. Aliquots of the medium and the filtrate were analyzed for PAH(9). When chlorphenol red was used slices were removed, blotted and smashed on glass slide for direct microscopic visualization. Nitrogen content of slices was determined by micro-Kjeldahl(10); dry weight by drying a minced slice first on steam bath and finally to constant weight over phosphorus pentoxide. Mitochondria were prepared by differential centrifugation of kidney homogenates in 0.25 M sucrose. Their capacity for oxidative phosphorylation (phosphorus/oxygen ratio) was measured(11) using $\alpha$-ketoglutarate as substrate. Phosphorus was estimated colorimetrically(12) and oxygen consumption determined manometrically.

*Results. Accumulation of PAH by slices:* On the basis of wet weight, slices of fetal kidney showed greater accumulation of PAH than did those of the neonatal animal. Increased concentration of PAH in the medium ($4.4 \times 10^{-4}$ M) did not alter these results.

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* This investigation supported by N.I.H. Division of Research Grants, postdoctoral fellowship, and Kidney Disease Fn.

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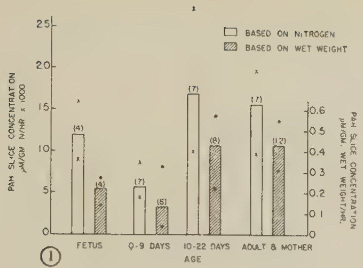

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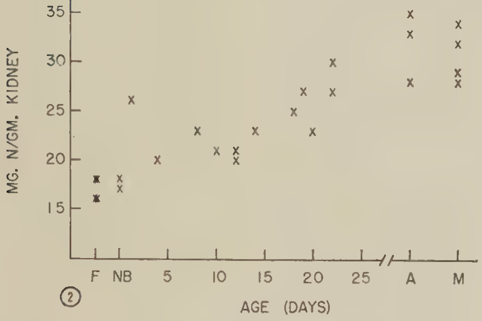

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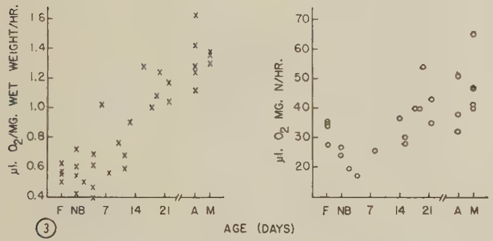

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FIG. 1. Relation of age to accumulation of *para*-aminohippurate by slices of kidney from rabbits. Slices of kidney 0.3-0.5 mm thick weighing from 80-200 mg were incubated in Warburg flasks in an atmosphere of oxygen for 60 min. at 30°C. The medium contained a modified Krebs Ringer phosphate solution at pH 7.4 and PAH in a final concentration of 7.4 × $10^{-5}$ M.

FIG. 2. Concentration of nitrogen in kidney of rabbit at various ages.

FIG. 3. Consumption of oxygen by slices of rabbit kidney related to age.

Slices of kidney from animals 10 days and older showed the same amount of PAH uptake as those from adult rabbits (Fig. 1).

Accumulation of PAH by slices of kidney from the fetus was less than that of the adult when based on wet weight of slice, but values for the fetus and adult were similar when based on nitrogen content. This result was related to progressive increase in nitrogen content of kidney with age (Fig. 2).

Uptake of PAH can be expressed by the ratio of its concentration in the slice to its concentration in the medium(6), *i.e.*,

$$\frac{\mu\text{moles PAH/g wet weight slice}}{\mu\text{moles PAH/ml medium}}.$$

All groups showed active accumulation as evidenced by an S/M ratio greater than 1. Kidneys from the newborn and from animals up to 9 days of age showed minimal accumulation of PAH. When slices of kidney were used from animals 10 days or older, ratios of 8 to 11 were obtained which were similar to those reported for adult rabbits(6). Dinitrophenol added to control flasks resulted in S/M ratios of less than 1 with all age groups, indicating that there is an energy requirement for uptake of PAH into slices. When the S/M ratio was based on nitrogen content or dry weight, *i.e.*,

$$\frac{\mu\text{moles PAH/g N or g dry wt of slice}}{\mu\text{moles PAH/ml medium}}$$

rather than wet weight of slice, the difference between values for the fetus and newborn was exaggerated while the difference between values from fetus and adult was decreased (Table I).

Droplets of chlorphenol red were visualized only in those preparations of tubular epithelial cells made from kidneys of animals 2 weeks of age or older.

*Oxygen consumption* by slices of rabbit kidney increased with age from newborn to adult whether based on nitrogen or wet weight of slice (Fig. 3). When based on nitrogen the lowest values for oxygen consumption were observed with slices of kidney from neonatal animals which also showed the least uptake of PAH. Other substrates, such as pyruvate, $\alpha$-ketoglutarate, and succinate did not increase consumption of oxygen by the slices. Per mg of nitrogen, the slice from fetal kidney con-

TABLE I. Accumulation of *para*-Aminohippurate by Slices of Kidney from Rabbits of Various Ages, Expressed as S/M Ratio.*

| | S/M per g | | |
|---|---|---|---|
| Age of rabbit | Wet wt | N | Dry wt |
| Fetus | 3.2 | 190 | 29 |
| 0- 8 days | 2.0 | 76 | 10 |
| 10-22 " | 10.2 | 330 | 46 |
| Adult | 8.4 | 250 | 39 |

* $\frac{\mu\text{moles PAH/unit wt of slice}}{\mu\text{moles PAH/ml medium}}$ after incubation of 60 min. at 30°C in oxygen.

sumed almost as much oxygen as the slice from the 10 to 21 day old rabbit.

*Phosphorus/oxygen ratios*: To determine whether differences in oxidative phosphorylation could account for the difference in PAH accumulation in slices from animals of various ages, mitochondrial phosphorus/oxygen ratios (P/O) were obtained. The P/O ratio in mitochondria isolated from kidneys of fetal, newborn, or adult rabbits was 1.9 to 2.9 indicating that decreased capacity for renal transport of PAH by neonatal animals does not result from uncoupling of oxygen and phosphorus.

*Discussion*. This study disagrees with previous reports which indicated a gradual continuous development of renal tubular function from fetal life to maturity. The data show that values of PAH accumulation *in vitro* for fetus and adult are similar when based either on dry weight or nitrogen content of kidney. The apparent difference in PAH accumulation between slices from the adult and fetal kidney (when based on wet weight) reflect an increased water content of the latter. Lack of visualization of CPR in slices of kidney from animals less than 2 weeks old may be the result of dilution of the dye. Tissue hydration does not, however, account for lack of accumulation of PAH in slices from the newborn kidney.

That oxygen consumption based on nitrogen content of the slice is lowest in the age group in which PAH accumulation is least may indicate some relationship between these 2 systems. Lack of enzymic development cannot explain a depressed accumulation of PAH by kidney of the newborn group, since the enzyme carrier system apparently is present in the fetus. Morphological development is gradual with no abrupt changes occurring at birth that might impair function(13-15). At present there is no explanation for the relative deficiency observed with slices of kidney in the newborn.

*Summary*. When the factor of high water content is eliminated, slices of kidney from the fetal rabbit accumulate as much PAH from surrounding medium as those from the adult. Slices of kidney from newborn rabbit are significantly deficient ($p = < .01$) in this function when compared to those from fetus or adult.

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# Prevention of Respiratory Decline in Necrotic Liver Degeneration by Antioxidants *in vitro*. (25319)

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The latent phase of dietary necrotic liver degeneration in the rat is characterized by respiratory decline, a metabolic lesion readily observed long before onset of gross pathological changes. Normal-appearing slices of livers of such animals are not capable of maintaining their initial oxygen consumption in the Warburg apparatus(1). The disturbance is *prevented* by feeding of factors which protect against liver necrosis, *i.e.*, Vit. E and Factor 3(1). It is also *reversed* by injection of Vit. E emulsions into the portal vein immediately prior to extirpation of the liver(2). In previous investigations with 13 different antioxidants it was shown that several of the compounds prevented dietary liver necrosis more or less effectively when fed in the diet, while others were completely inactive(3). The same antioxidants were then tested for capacity to reverse the tendency to respiratory decline after injection into the liver *via* portal vein(4); by this method almost the same order of relative potencies was obtained. DPPD (N, N′-diphenyl-p-phenylenediamine) was most effective. By the portal route it was approximately twice as potent as D,L-α-tocopherol in reversing the tendency to respiratory failure. The current paper deals with prevention of respiratory decline by direct *in vitro* incubation of liver slices with the antioxidants. D,L-α-tocopherol or its esters leave respiratory decline completely unaffected when added to the Warburg medium. Two tocopherol metabolites, however, isolated by Simon *et al.*(5) from urines of rabbits and humans, possessed high *in vitro* activity(6). We discovered that synthetic antioxidants are also effective when applied to liver slices directly. A quantitative comparison *in vitro* of the 13 antioxidants screened previously in the diet and by the intraportal technic is given below.

*Methods.* Most experimental details have been described(4). Weanling rats of inbred Fischer 344 strain were maintained on 30% *Torula* yeast diet(7). Under our conditions, this ration produces necrotic liver degeneration with practically 100% incidence, after average survival time of 21 days. Animals for Warburg experiments were taken between 14th and 17th day, at average weight of 45 g. Liver necrosis was seen occasionally at that time; only normal-appearing liver was used for testing. Rats were sacrificed by decapitation, and approximately 400 mg of slices were prepared by hand from the left lateral liver lobe, divided into 4 equal portions of 90 mg (± 15 mg) and used in 4 different flasks. On the average, 6 minutes were required for killing and preparation of the slices. Three ml of oxygenated Krebs-Ringer phosphate buffer/flask was used, with .01 M glucose as substrate. Of 4 flasks containing slices from one liver, 3 received supplements at different doses; the other served as control. Incubation was carried out under $O_2$ at 37.5° for 140 minutes. *Evaluation.* The formula for calculation of respiratory decline, *i.e.*, failure of liver slices to maintain initial $O_2$ consumption, has been given previously(4). The effect of supplements is expressed as per cent *prevention* of respiratory decline. It is calculated by comparing the decline of supplemented slices (dB) to the decline of their respective controls (dA), using the formula $P = 100 - (dB \times 100)/dA$. *Antioxidants.* The compounds used are listed in the Tables. Water insoluble substances were dissolved in ethanol to make a 10% solution. A portion of the latter was emulsified in incubation medium with a Potter-Elvehjem homogenizer. Of this emulsion, the required amount was added to the flask. The amount of ethanol present in 3 ml of medium was thus roughly 10 times that of the antioxidant. With one exception (hydroquinone) it never exceeded

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* Studies performed in 1955 during tenure of a Brewer's Yeast Council Research Fellowship.

.002 ml; for most experiments it was much less.

*Results. Control experiments.* It is well established that liver slices from normal, unfasted rats maintain their $O_2$ uptake in the Warburg apparatus at or close to initial values for 4 to 6 hours(1). In livers of rats on complete diets respiratory decline is practically zero, while in rats on 30% *Torula* yeast diet it amounts (on the average) to *ca* 75% of initial rate of $O_2$ consumption after 90-120 minutes. To ascertain that results were not affected by non-enzymatic oxidation of the supplements, the 2 compounds most likely to react directly with $O_2$, ascorbic acid and hydroquinone, were incubated in buffer in the absence of slices. $O_2$ uptake was measurable, but negligible as compared to that of liver slices. With 400 $\mu$g of hydroquinone, the value was 6 $\mu$l/hour. These findings do not, of course, exclude the possibility that the compounds could stimulate $O_2$ consumption in the presence of slices. It is noted in the Tables that average initial respiration (0-30 min. interval) is usually somewhat higher with the supplement (column B) than without (column A), but the differences are small and most likely caused by an effect of the supplement on the incipient respiratory decline during the first 30-minute interval.

The effect of DPPD on slices of intact liver was studied with animals protected against necrotic liver degeneration by 50 mg% D, L-$\alpha$-tocopheryl acetate and .12% D, L-methionine in their diet. Decline of $O_2$ consumption after 2 hours incubation was not more than 6%, and *in vitro* addition of an optimal dose of DPPD, was without any demonstrable influence on respiration (Fig. 1A). In simultaneous experiments with deficient liver slices (Fig. 1B), the breakdown of respiration was prevented by the supplement. Addition of .02 ml of ethanol alone to deficient slices had no significant influence except for slight stimulation of initial $O_2$ consumption. When lower amounts were used, the pattern of respiration was not different from that of controls (Table I).

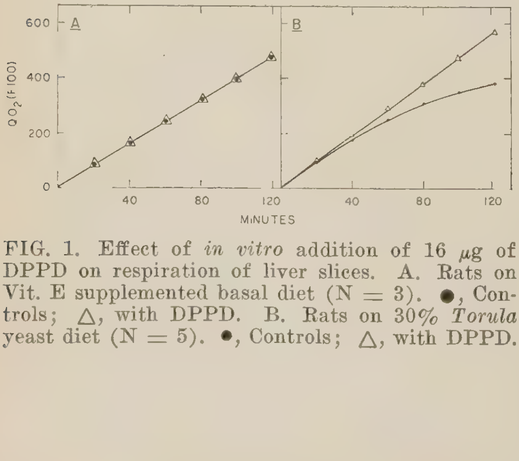

FIG. 1. Effect of *in vitro* addition of 16 $\mu$g of DPPD on respiration of liver slices. A. Rats on Vit. E supplemented basal diet (N = 3). ●, Controls; △, with DPPD. B. Rats on 30% *Torula* yeast diet (N = 5). ●, Controls; △, with DPPD.

*Effect of antioxidants on respiratory decline.* As in the 2 preceding publications(3,4) the compounds used are grouped in 3 categories to facilitate organization of the data. Group 1 consists of ascorbic acid, methylene blue and Antabuse, which can act as antioxidants but are better known for other properties. Substances of phenolic character (NDGA, n-Propylgallate, DBPC, BHA, Propylparasept, hydroquinone) which were ineffective in the preceding tests form Group II. Group III contains compounds (di-tert-amyl-hydroquinone, Santoquin, Santoflex B and DPPD) which protect effectively against dietary necrotic liver degeneration(3) and also reverse respiratory decline when injected intraportally(4). Of the compounds of Group I, ascorbic acid was relatively ineffective *in vitro* (Table I). Antabuse afforded more protection, but its activity was much below that of methylene blue. The dye produced 50% prevention of metabolic lesion when roughly 1 $\mu$g was added/flask. This potency *in vitro* is in distinct contrast to the behavior of the compound *in vivo, i.e.,* in dietary and intraportal tests. When injected into the portal vein, methylene blue was rapidly decolorized after reaching the liver and it did not reverse the tendency towards respiratory failure.† When added *in vitro,* on the other hand, the blue color was maintained and respiratory decline was prevented.

Of compounds of Group II, hydroquinone and Propylparasept were ineffective, while a

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† The current study evolved from a chance observation: In one instance, during intraportal injection experiments, methylene blue remained unchanged in a liver lobe, possibly due to blocking of circulation after operation. Slices from this lobe maintained normal respiration, whereas other, decolorized lobes of liver demonstrated respiratory failure.

TABLE I. *In Vitro* Effect of Antioxidants on Respiratory Decline of Liver Slices. Group I (ascorbic acid, methylene blue, Antabuse) and Group II (phenolic antioxidants).

| Dose in $\mu$g/flask | No. of exp. | $Qo_2$ (F100)* A Control 0-30 min. | A Control 100-120 min. | B With supplement 0-30 min. | B With supplement 100-120 min. | % respiratory decline† dA | % respiratory decline† dB | Prevention of respiratory decline‡ in % |
|---|---|---|---|---|---|---|---|---|
| | | | | Controls (ethanol) | | | | |
| 1584§ | 6 | 277 | 61 | 296 | 57 | 79 | 81 | 0 (−3.5 ± 2.6)‖ |
| 50 | 4 | 256 | 62 | 259 | 65 | 76 | 75 | 0 ( 1 ± 1.9) |
| | | | | Ascorbic acid | | | | |
| 100 | 4 | 279 | 77 | 277 | 109 | 73 | 61 | 15 ± 4.1 |
| 400 | 5 | 286 | 68 | 318 | 165 | 75 | 48 | 37 ±14.6 |
| | | | | Methylene blue chloride | | | | |
| .4 | 5 | 274 | 82 | 278 | 124 | 70 | 56 | 24 ±10.2 |
| 1.65 | 5 | 280 | 84 | 301 | 215 | 70 | 28 | 62 ± 8.1 |
| 6.25 | 5 | 328 | 57 | 352 | 254 | 83 | 28 | 65 ± 7.4 |
| 25 | 5 | 317 | 65 | 353 | 300 | 79 | 14 | 83 ± 2.2 |
| | | | | Antabuse (tetraethylthiuram disulfide) | | | | |
| 6.25 | 5 | 320 | 75 | 350 | 121 | 76 | 65 | 17 ±10.1 |
| 25 | 5 | ” | ” | 364 | 178 | ” | 51 | 34 ± 9.6 |
| 100 | 5 | ” | ” | 346 | 251 | ” | 27 | 64 ±11.0 |
| | | | | NDGA (nordihydroguaiaretic acid) | | | | |
| 6.25 | 5 | 301 | 109 | 340 | 119 | 64 | 64 | 0 (−2 ± 6.2) |
| 25 | 5 | ” | ” | 335 | 191 | ” | 40 | 49 ±19.0 |
| 100 | 5 | 307 | 50 | 322 | 196 | 84 | 37 | 58 ±12.9 |
| | | | | n-Propylgallate (propylester of gallic acid) | | | | |
| 6.25 | 5 | 342 | 89 | 370 | 125 | 75 | 67 | 12 ± 5.1 |
| 25 | 5 | ” | ” | 389 | 179 | ” | 55 | 27 ±11.2 |
| 100 | 5 | ” | ” | 378 | 191 | ” | 50 | 35 ± 9.0 |
| | | | | DBPC (2,6-di-tert-butyl-4-methylphenol) | | | | |
| 1.56 | 5 | 301 | 88 | 357 | 150 | 74 | 59 | 20 ± 6.8 |
| 6.25 | 5 | 278 | 74 | 328 | 140 | 76 | 57 | 25 ±10.7 |
| 25 | 5 | 313 | 101 | 353 | 225 | 68 | 37 | 49 ± 6.3 |
| 100 | 4 | 307 | 104 | 325 | 271 | 66 | 14 | 80 ±12 |
| | | | | BHA (2- and 3-tert-butyl-4-methoxyphenol) | | | | |
| 6.25 | 5 | 293 | 66 | 324 | 83 | 78 | 75 | 0 (4 ± 4.8) |
| 25 | 5 | ” | ” | 344 | 177 | ” | 48 | 39 ± 6.8 |
| 100 | 5 | 292 | 53 | 325 | 283 | 82 | 12 | 85 ± 2.4 |
| | | | | Propylparasept (propylester of p-hydroxybenzoic acid) | | | | |
| 100 | 5 | 301 | 70 | 325 | 51 | 80 | 85 | 0 (−8 ± 9.3) |
| | | | | Hydroquinone | | | | |
| 400 | 5 | 303 | 76 | 319 | 101 | 74 | 69 | 0 (5 ± 5.4) |

* $O_2$ consumption in $\mu$l/hr and 100 mg of liver slices; values calculated for first 30 min. interval and for 100-120 min. interval, respectively.

† $Qo_2$ (0-30) − $Qo_2$ (100-120), expressed in % of $Qo_2$ (0-30).

‡ Prevention = dA minus dB, expressed in % of dA.

§ Equal to .002 ml.

‖ Mean ± stand. error of mean.

measurable degree of prevention was obtained with high levels of NDGA, Propylgallate, DBPC and BHA (Table I). The approximate dose required for 50% protection ranged from 25 to more than 100 $\mu$g. DBPC was the most active of the compounds; this is in good agreement with results of intraportal study, where DBPC showed a moderate effect, while the other 5 compounds were without influence at dose levels tested. In the dietary test against liver necrosis, all 6 of these antioxidants had been inactive.

The antioxidants of Group III, which prevented liver necrosis more or less effectively and also reversed respiratory decline upon intraportal injection, proved to be the most

TABLE II. *In Vitro* Effect on Respiratory Decline. Group III (active compounds and vitamin E).

| | | $Q_{O_2}$ (F100)* | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | A Control | | B With supplement | | % respiratory decline† | | |
| Dose in $\mu$g/flask | No. of exp. | 0-30 min. | 100-120 min. | 0-30 min. | 100-120 min. | dA | dB | Prevention of respiratory decline‡ in % |
| Amylhydroquinone (2,5-di-tert-amylhydroquinone) | | | | | | | | |
| 1.56 | 5 | 280 | 50 | 312 | 91 | 83 | 71 | 14 ± 6.9‖ |
| 6.25 | 5 | ” | ” | 334 | 274 | ” | 17 | 79 ±10.8 |
| 25 | 5 | ” | ” | 307 | 273 | ” | 9 | 88 ± 7.9 |
| Santoquin (6-ethoxy-1,2-dihydro-2,2,4-trimethylquinoline) | | | | | | | | |
| 1.56 | 5 | 315 | 74 | 352 | 155 | 76 | 56 | 28 ± 9.7 |
| 6.25 | 5 | 307 | 76 | 332 | 278 | ” | 15 | 79 ± 7.2 |
| 25 | 5 | ” | ” | 350 | 287 | ” | 17 | 76 ± 4.6 |
| Santoflex B (6-phenyl-1,2-dihydro-2,2,4-trimethylquinoline) | | | | | | | | |
| .0625 | 5 | 344 | 99 | 355 | 110 | 74 | 69 | 7 ± 2.7 |
| .25 | 5 | ” | ” | 353 | 154 | ” | 57 | 23 ± 5.3 |
| 1 | 5 | ” | ” | 358 | 293 | ” | 19 | 75 ± 4.0 |
| DPPD (N,N′-diphenyl-p-phenylenediamine) | | | | | | | | |
| .0156 | 5 | 269 | 59 | 271 | 72 | 78 | 74 | 5 ± 3.4 |
| .0625 | 5 | 329 | 79 | 319 | 149 | 76 | 52 | 32 ± 5.1 |
| .25 | 5 | ” | ” | 330 | 255 | ” | 23 | 70 ± 5.0 |
| 1 | 5 | ” | ” | 344 | 319 | ” | 7 | 91 ± 1.4 |
| 16 | 5 | 274 | 82 | 288 | 274 | 70 | 2 | 98 ± 2.2 |
| D,L-α-tocopherol | | | | | | | | |
| 50 | 5 | 333 | 94 | 364 | 91 | 72 | 77 | 0 (−6 ± 6.5) |
| 200 | 5 | ” | ” | 350 | 106 | ” | 70 | 0 ( 4 ± 5.2) |
| D-α-Tocopheryl Polyethylene Glycol-1000 Succinate | | | | | | | | |
| 1000 | 5 | 275 | 58 | 273 | 68 | 79 | 74 | 7 ± 5.3 |
| Tocopherol metabolite§<br>2-(3-hydroxy-3-methyl-5-carboxypentyl)-3,5,6-trimethylbenzoquinone | | | | | | | | |
| 1.56 | 5 | 251 | 87 | 290 | 145 | 66 | 51 | 27 ± 8.6 |
| 6.25 | 5 | 228 | 60 | 258 | 161 | 73 | 39 | 50 ±20.6 |
| 25 | 4 | 243 | 69 | 294 | 256 | 71 | 13 | 83 ± 4.4 |

* $O_2$ consumption in $\mu$l/hr and 100 mg of liver slices; values calculated for first 30 min. interval and for 100-120 min. interval, respectively.

† $Q_{O_2}$ (0-30) − $Q_{O_2}$ (100-120), expressed in % of $Q_{O_2}$ (0-30).

‡ Prevention = dA minus dB, expressed in % of dA.

§ Published previously(6).

‖ Mean ± stand. error of mean.

active compounds in preventing the metabolic lesion *in vitro* as well (Table II). While unsubstituted hydroquinone did not prevent respiratory decline *in vitro*, the substituted, sterically hindered amylhydroquinone proved quite potent. Two quinoline derivatives, Santoquin and Santoflex, also produced a response analogous to that found in *in vivo* assay systems: Santoquin, the 6-ethoxy substituted compound, was slightly less effective than Santoflex, the 6-phenyl form. Of the first, *ca* 3.5 $\mu$g was required for 50% prevention, whereas *ca* 0.6 $\mu$g of the latter gave the same effect. Activity of Santoquin seems to reach a ceiling at *ca* 6 $\mu$g; 4 times this dose did not elicit a higher degree of response.

As in previous studies, DPPD was the most active member of the series. As little as .06 $\mu$g, representing *ca* .00025 $\mu$mole of DPPD, gave a significant degree of protection, and 1 $\mu$g prevented decline almost completely. If this amount were directly oxidized, it would account for less than .01 $\mu$l of $O_2$ consumed. In the presence of .06 $\mu$g of DPPD, respiration of liver slices in the last 20 minute interval alone was approximately 23 $\mu$l higher than without supplement, which shows clearly that the phenomenon cannot be interpreted as a substrate effect.

In accordance with previously published results(6), 50 and 200 $\mu$g of D,L-α-tocopherol, added *in vitro*, did not prevent respiratory decline. This cannot be explained on the assumption of poor solubility; water soluble tocopheryl-polyethylene glycol succinate, highly effective *in vivo,* was equally without effect when 1000 μg were given *in vitro.* The tocopherol metabolite, 2-(3-hydroxy-3-methyl-5-carboxypentyl) - 3,5,6-trimethylbenzoquinone, on the other hand, prevented respiratory failure quite effectively, even though given in the quinone form(6).

*Discussion.* Comparing these results with those published previously, it is evident that the 13 antioxidants studied produce a similar pattern of response in all 3 test systems. They offer protection against dietary necrotic liver degeneration when fed in the diet(3), reversal of respiratory decline of liver slices upon intraportal injection(4), and prevention of respiratory decline by *in vitro* addition. While earlier studies showed that the site of action was within the organism, the present evidence demonstrates that the effect occurs directly in liver tissue. Since *in vitro* technic is more sensitive and permits study of wider dose ranges, it shows several substances capable of protection against the metabolic lesion which formerly were found inactive. Differences in effective dose levels of various substances are large, but with 2 main exceptions the relative positions of the compounds on the scale of potencies remain unchanged. The exceptions are Vit. E and methylene blue.

Vit. E and its esters, while superior to DPPD in the diet, and almost equal to DPPD in the intraportal injection, are completely inactive *in vitro.* This observation led to the concept that tocopherol is converted in the organism into an "active form"(8), with the subsequent discovery that the 2 Vit. E-metabolites, 2-(3-hydroxy-3-methyl-5-carboxy)-pentyl-3,5,6-trimethyl-benzoquinone and its γ-lactone, are active in the *in vitro* system(6).

Methylene blue, on the other hand, is *in vitro* comparatively much more potent than *in vivo;* it moves up into third place behind DPPD and Santoflex B. This shift is related to the fact that in the aerobic Warburg medium the dye is maintained in the oxidized state, whereas *in vivo* it is readily reduced to the leuco-form.

With reference to respiratory decline and death from liver necrosis, it is clear that the effective compounds of our antioxidant series truly replace Vit. E, or rather its active form, in its function within liver tissue. (In studies with DPPD and resorption sterility of the rat, Draper *et al.* have come to similar results (9)). As discussed previously, a certain degree of structural specificity governs metabolic efficiency of the compounds. For example, hydroquinone is inactive but is converted into a potent compound by substitution of amyl groups in the 2 and 5 positions. This indicates the importance of sterical hindrance for the effect.

Since quantities effective in preventing respiratory decline are too small to yield an effect as substrates, most, if not all of the compounds must act on the catalytic level. The exact site and mode of action of the substances in the liver cell cannot be deduced from the data at hand. It is obvious that physicochemical properties essential for this effect are also important for antioxidant-activity, but there is no parallelism of antioxidant potencies of compounds with their capacity to influence the respiratory lesion. It is probable that the antioxidant function proper, *i.e.,* protection against consequences of peroxide formation, is not involved in prevention of respiratory decline, since a number of the compounds with excellent, antioxidant capacity are ineffective in all 3 test systems even at large dose levels. The most important argument against the simple antioxidant theory of action is derived from the behavior of tocopherol itself. The vitamin is known to have strong antioxidant properties, and yet is completely ineffective on our *in vitro* system (while effective in the diet and after intraportal injection). Vit. E metabolites, on the other hand, are potent agents in prevention of respiratory decline, even though they are used in the quinone form which is worthless as antioxidant. One has to consider the possibility that the active compounds could participate catalytically in some specific aspect of electron transfer which happens to require structural and functional molecular properties similar to those of antioxidants.

*Summary.* The effect of 13 antioxidants on liver slices during the latent phase of necrotic

liver degeneration, was studied *in vitro*. The characteristic respiratory decline, *i.e.*, breakdown of $O_2$ consumption in the Warburg apparatus, was prevented by addition of most compounds at catalytic dose levels. With increasing amounts of compounds, dose response curves were obtained. The scale of relative potencies *in vitro* followed closely that previously established *in vivo* for protection against liver necrosis by feeding of the compounds, as well as for reversal of respiratory decline by intraportal injection, with the exception of Vit. E and methylene blue. Tocopherol and its esters were completely inactive *in vitro,* while the tocopherol metabolite showed high potency in preventing the metabolic lesion. Methylene blue, inactive in the *in vivo* systems, was highly potent in the Warburg assay. The most potent substance, DPPD, produced a significant effect with .06 $\mu$g/100 mg of liver tissue in 3 cc medium. Santoquin, Santoflex B and amylhydroquinone were effective at doses from less than 1 to 6 $\mu$g. Less protection was found with phenolic compounds, such as NDGA, Propylgallate, DBPC and BHA; hydroquinone and Propylparasept were without activity. It is concluded that the substances act directly in the liver cell, perhaps not merely as antioxidants but as catalysts in intermediary metabolism.

For samples of antioxidants we are indebted to Ayerst Labs., N. Y., Nordigard Corp., Chicago; Heyden Chemical Corp., N. Y.; Koppers Co.; Pittsburgh; Eastman Chemical Products, Kingsport, Tenn.; Shell Chemical Corp., N. Y., Monsanto Chemical Co., St. Louis; and the B. F. Goodrich Chemical Co., Cleveland, O.

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## Comparison of Normal and Buffered Saline Solution for Fluid Replacement Following Tourniquet Shock.* (25320)

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Most experimental work dealing with electrolyte solution replacement after tourniquet trauma has been done with isotonic NaCl solution. The parenteral or oral use of adequate amounts of such solution protects against an otherwise lethal tourniquet trauma(1,2). Speculation on whether the therapeutic effect of saline derives from Na or Cl ion or both. is not new. Allen(3) reported that in treatment of tourniquet shock, normal saline gave better survival results than solutions more alkaline or more acid. He suggested that to combat acidosis, which accompanies shock, by alkaline solutions might actually be deleterious. Our method(1) of intravenous infusion in therapy of tourniquet trauma lends itself to a study of this problem. Our experiments were undertaken to compare survival rates, after an otherwise lethal tourniquet trauma, when animals were treated with isotonic NaCl or an alkaline buffered saline solution as used by Moyer(4). Induced pH and electrolyte changes were also measured.

*Methods.* Male albino rats of Holtzman Farms, weighing 200-250 g were used. Each animal was individually housed in air-condi-

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* Supported by Dept. of Army, Office of Surgeon General.

tioned laboratory for at least 4 days before use. Food was removed from cages approximately 15 hours before application of tourniquets(3). On morning of experiment, all animals were weighed, anesthetized and bilateral hind limb tourniquets applied for 4½ hours. This duration of trauma has been previously shown to be lethal to at least 96% of untreated controls in 200-250 g weight range. At time of tourniquet release, animals were again anesthetized and a polyethylene catheter introduced into the right jugular vein for administration of 1 of 2 therapeutic solutions.

| | |
|---|---|
| Sol. 1 | |
| Isotonic NaCl (pH 6.14) | 154 meq $Na^+$/l<br>154 meq $Cl^-$/l |
| Sol. 2 | |
| Saline bicarb. mixture (pH 8.12) | 129 meq $Cl^-$/l<br>25 meq $HCO_3^-$/l |
| 154 meq $Na^+$/l | 154 meq/l |

Infusion was begun 1 hour after release of tourniquets and rate of infusion so timed that 5 ml of solution was administered/100 g of body weight for 3 hours ±17 minutes. Infusions were given simultaneously by 2 or 3 variable speed constant infusion pumps, each having places for 12 syringes. At completion of infusion, the vein was ligated and the neck closed with skin clips. Then the animals were returned to their cages where food and water were available to them. Survival was recorded at 24 and 48 hours after tourniquet release. pH measurements were made (Beckman blood electrode pH meter) in 2 animals in each group at conclusion of infusion. In a separate experiment the serum electrolyte pattern was determined in both groups at conclusion of therapy. This was also done in appropriate untreated controls. Measurements of serum Na, K, $CO_2$ combining power and Cl were made. Blood for these measurements was withdrawn *via* a polyethylene catheter placed in carotid artery. $CO_2$ combining power was determined on Kopp Natelson Microgasometer. Chlorides were determined by method of Schales and Schales(5). Na and K levels were measured in internal standard flame photometer. These determinations were made in each of the following groups: Group I animals bled 1 hour after tourniquet release. Group II animals bled 4 hours after tourniquet release (control). Group III animals bled 4 hours after tourniquet release, having received 5 ml of isotonic saline solution/100 g body weight beginning at 1 hour and ending at 4 hours after tourniquet release. Group IV animals were bled 4 hours after tourniquet release, having received 5 ml saline-bicarbonate solution/100 g body weight beginning at 1 hour and ending at 4 hours after tourniquet release.

*Results.* Comparison of therapeutic effectiveness showed a slightly higher percentage of survival in Group III (isotonic sodium chloride group) (Table I). Chi square analysis of these data shows the difference not to be statistically significant ($p > 0.10$).

Electrolyte determinations showed little difference between untreated control animals at one and 4 hours after tourniquet release. In groups treated with isotonic sodium chloride and saline-bicarbonate solutions, the serum electrolyte concentration was very much the same as in untreated groups, with the exception of a distinct elevation of serum chloride of the same degree in both groups, and a suggestive elevation of serum sodium in the bicarbonate-saline group.

*Discussion.* It has been previously demonstrated(1,2) that use of oral or parenteral electrolyte solutions in adequate amounts protects against the otherwise lethal effect of certain standardized plasma loss traumas (tourniquets, burns).

Rosenthal(6) was able to demonstrate no difference in therapeutic effectiveness between various Na solutions used in treatment of burns in mice. Moyer(4), however, using solutions tested in experiments in pre-burn treatments in dogs, demonstrated significantly longer survival time in a group treated with saline-bicarbonate mixture than in a group treated with isotonic sodium chloride solution.

The present study demonstrates that NaCl and saline-bicarbonate solutions have the same degree of protection as measured by survival rates (Table I). Further, the solutions produced an equivalent increase in serum chloride compared to controls. There was no demonstrable effect on $CO_2$ combining power.

*Summary.* 1. Simultaneous comparison

TABLE I. Survival following Intravenous Infusion of 5 ml/100 g Body Weight following 4½ Hr Bilateral Hind Limb Tourniquet Application.

| | | 24 hr | | | 48 hr | | | |
|---|---|---|---|---|---|---|---|---|
| | Type infusion | Lived | Died | % survival | Lived | Died | % survival | pH at conclusion of infusion |
| Exp. 1 | .9% NaCl | 8 | 2 | | 7 | 3 | | 7.40, 7.43 |
| | Saline bicarbonate | 8 | 2 | | 5 | 5 | | 7.53, 7.43 |
| | Untreated control | 2 | 2 | | 0 | 4 | | |
| 2 | .9% NaCl | 9 | 8 | | 5 | 12 | | |
| | Saline bicarbonate | 8 | 10 | | 4 | 14 | | |
| | Untreated control | 0 | 4 | | 0 | 4 | | |
| Pooled results | .9% NaCl | 17 | 10 | 63.0 | 12 | 15 | 44.4 | |
| | Saline bicarbonate | 16 | 12 | 57.3 | 9 | 19 | 32.1 | |
| | Untreated control | 2 | 6 | 25 | 0 | 8 | 0 | |

was made of therapeutic effectiveness of isotonic NaCl and saline-bicarbonate solutions when used in treatment of an otherwise lethal tourniquet trauma. The method of treatment allowed slow constant intravenous infusion of therapeutic solutions over period of greatest loss of fluid from circulating volume into traumatized extremities. The solutions were equally effective in increasing survival rate over that of untreated, traumatized controls. 2. Both solutions produced the same alteration in serum electrolyte concentration, *i.e.*, elevation of serum Cl values. The lowered $CO_2$ combining power of untreated control was not appreciably altered in the treatment groups.

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## Effect of Relaxin on Mammary Gland Growth in the Female Rat.[*] (25321)

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All previous studies concerning the role of relaxin in growth of mammary gland in the rat have suggested that relaxin acts as a potentiator (or synergist) of lobule-alveolar growth in conjunction with estrogen and progesterone(1,2,3,4). Recently, in studies using desoxyribosenucleic acid (DNA) as a measure of mammary gland growth in both intact and gonadectomized male and female mice, it was shown that estrogen and relaxin alone are capable of stimulating increases in DNA associated with morphological evidence of marked lobule-alveolar growth(5,6). These and previous studies suggested that estrogen and progesterone stimulate endogenous secretion of relaxin and that relaxin might be the agent which stimulates increased secretion of pituitary mammogen rather than progesterone, since it was shown that estrogen and relaxin were ineffective in hypophysectomized mice. The present study extends our studies on the role of estrogen and relaxin in stimulating lo-

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TABLE I. Effect of Relaxin on Mammary Gland Growth and Pituitary Weight in the Female Rat.

| Group | Treatment, amt × days | No. of rats | Body wt (g) | Pituitary | | Mammary gland | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Wet wt (mg) | Wet wt (mg) 100 g body wt | DFFT wt (mg) | DNA (μg) mg of DFFT | Total DNA (mg) | Total DNA (mg) 100 g body wt |
| Intact (1) | None—Control | 10 | 281 ± 4 | 12.8 ± .5 | 4.6 ± .2 | 303.2 ± 8.6 | 28.8 ± 1.0 | 8.769 ± .481 | 3.110 ± .161 |
| (2) | EB, 1 μg × 7 | " | 279 ± 6 | 13.3 ± .7 | 4.8 ± .2 | 329.4 ± 15.6 | 27.8 ± 1.5 | 9.220 ± .778 | 3.298 ± .243 |
| (3) | EB, 1 μg × 7<br>R, 15 GPU | " | 286 ± 5 | 14.8 ± .5* | 5.2 ± .1* | 330.8 ± 17.5 | 32.8 ± .9* | 10.885 ± .742* | 3.793 ± .232* |
| (4) | EB, 1 μg × 7<br>R, 45 GPU | " | 262 ± 5 | 15.0 ± .8* | 5.7 ± .3† | 364.3 ± 9.9‡ | 29.8 ± .9 | 10.846 ± .316† | 4.149 ± .166‡ |
| Castrated (5) | None—Control<br>" | 10<br>15 | 279 ±11<br>273.7 | 12.4 ± .3 | 4.5 ± .3 | 356.1 ± 21.0 | 25.9 ± .7<br>23.2 ± .54 | 9.261 ± .604 | 3.320 ± .183<br>3.05 ± .14 § |
| (6) | EB, 1 μg × 9 | 10 | 281 ± 4 | 14.8 ± .5† | 5.3 ± .2* | 326.7 ± 15.6 | 26.6 ± 1.2 | 9.162 ± .255 | 3.109 ± .095 |
| (7) | EB, 1 μg × 9<br>R, 15 GPU | 11 | 240 ± 3 | 14.0 ± .5* | 5.9 ± .3† | 342.9 ± 10.8 | 26.3 ± .5 | 9.006 ± .219 | 3.760 ± .097* |
| (8) | EB, 1 μg × 9<br>R, 45 GPU | 10 | 273 ± 4 | 15.3 ± .6† | 5.6 ± .2† | 273.1 ± 12.8 | 37.0 ± 1.16‡ | 10.545 ± .672 | 3.852 ± .222 |

EB = Estradiol benzoate. R = Relaxin. DFFT = Dry, fat-free tissue. Values are means ± S.E.
* Significant at 5% level to control. † Significant at 1% level to control. ‡ Significant at .1% level to control. § Data from Moon *et al.*(7).

bule-alveolar growth in the intact and ovariectomized rat.

*Materials and methods.* Adult female rats of Sprague-Dawley-Rolfsmeyer strain weighing approximately 230 to 250 g, kept in a room artificially illuminated during daylight hours, at a uniform temperature of 78 ± 1° F, were fed commercial rat feed. One lot was ovariectomized 2 weeks before start of experiment. Estradiol benzoate (E.B.) was injected daily in 1 μg amounts for 7 days to intact rats and for 9 days to ovariectomized rats concomitant with 30 and 90 GPU of relaxin suspended in 0.1 ml sesame oil plus 5% beeswax carrier every other day.‡ One day following final injection, animals were sacrificed, 6 abdominal-inguinal glands removed and frozen for 4 days. The 4 anterior glands were prepared for whole mount examination. Tissues were prepared for DNA determination by method previously described(7). The pituitaries were weighed and frozen until assayed for mammogenic activity. Mammogenic potency of rat pituitaries was assayed in 19 g ovariectomized mice. Each pituitary was ground in cool agate mortar and suspended in 0.6 ml normal saline. Each mouse received ½ rat pituitary/day subcutaneously for 10 days. On 11th day glands were removed for DNA determination (using 30 mg sample) and whole mount examination made of glands.

*Results.* Indices of mammary gland growth stimulated by the hormones involves increase in dry-fat-free tissue (DFFT), DNA/mg DFFT, total DNA/6 glands, and total DNA/100 g body weight (Table I). Increase in DFFT in intact animals was associated with hormone administration. The rats given estrogen and the higher level of relaxin had glands significantly heavier than the controls (at 0.1% level). It will be noted also that total DNA/6 glands increased significantly above controls as relaxin was increased (at 5 and 1% level respectively) and when compared on body weight basis, the difference at higher level of relaxin became significant at the 0.1% level.

Morphological observations of whole

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‡ Relaxin preparation kindly supplied by Dr. R. L. Kroc, Chilcott Lab., Morris Plains, N. J., assayed 30 GPU/mg.

mounts of glands indicated chiefly duct development in the controls and those receiving estrogen alone, whereas those on estrogen and relaxin showed increased lobule-alveolar growth.§ In general, the extent of lobule development was related to the mean total DNA in the 2 groups.

In the ovariectomized groups similar but less significant differences were noted in comparison to intact rats. This may be due in part to the relatively high total DNA values for the castrate-control rats (3.32 mg/100 g body wt.) in comparison to the intact control rats (3.11 mg) and to a control group of 15 similar animals in a separate but concurrent experiment(7) with mean total DNA/100 g body wt. of 3.05 mg. Based on total DNA/100 g body weight of control rats, only the increase in DNA of rats on 15 GPU relaxin/day was significant (at 5% level). However, if the control value for ovariectomized rats reported previously is used (3.05 mg), then the 15 GPU rats are significantly increased at the 1% level and the 45 GPU rats are significant at the 5% level. Less lobule-alveolar development was noted in these glands as compared to glands of the intact groups.

The less significant lobule-alveolar growth in the rats receiving estrogen and relaxin following ovariectomy in comparison to the corresponding intact rats is believed to be due, in part, to the time lag in restoring DNA of glands to normal after involution resulting from ovariectomy, but it may be due to possible hormonal contribution supplied by intact ovaries.

The influence of estrogen alone and concurrent relaxin on pituitary weight is shown (Table I). Relaxin and estrogen stimulated significant increases in weight in comparison with control values.

No evidence of mammogenic activity in the pituitaries of rats under test was shown by ½ pituitary/day for 10 days in mice. Total DNA of mice mammary glands was not increased significantly.

*Discussion.* Our study with female rats confirms our previous work in male and female mice concerning the influence of relaxin and estrogen in stimulating growth of the mammary lobule-alveolar system. It was previously shown(7) that 1 μg estradiol benzoate and 2 mg progesterone injected into mature ovariectomized rats for 19 days resulted in mammary gland growth (mean total DNA/100 g) equal to that of rats pregnant for a similar period. In unpublished work currently in progress, it has been shown that estradiol benzoate 1 μg and 15 GPU relaxin for 19 days had induced mean total DNA/100 g increase comparable to that induced by 1 mg progesterone. If estrogen and progesterone synergize to stimulate endogenous secretion of relaxin which, in turn, stimulates mammary gland growth, it would indicate that approximately 15 GPU of relaxin/day may be stimulated by 1 mg progesterone/day.

*Summary.* 1. Intact and ovariectomized rats injected with 1 μg estradiol benzoate and 15 and 45 GPU relaxin stimulated significant growth of lobule-alveolar system in about 10 days as indicated by total DNA/6 glands/100 g and morphological observations. Thus, relaxin need no longer be considered merely a synergist of estrogen and progesterone in mammary gland growth in rats as previously suggested.

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§ It should be pointed out that the mature virgin rat duct system contains large numbers of branching ducts in contrast to the virgin mouse). It is far more difficult in the rat to differentiate between ducts and lobule-alveoli than in the mouse.

# Diet and Cholesteremia. IV. Effects of Carbohydrate and Nicotinic Acid.* (25322)

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Serum cholesterol concentrations of rats fed a hypercholesteremic diet with sucrose as carbohydrate, are reported to exceed those of comparable animals fed a similar diet with starch as the carbohydrate(1). Also, in preliminary study, the substitution of wheat flour for sucrose at a level of 29.5% in a hypercholesteremic diet caused some reduction in serum cholesterol concentration in the rat. Since wheat gluten in the diet alleviated hypercholesteremia(2), it was not clear whether reduction in hypercholesteremia from feeding wheat flour was attributable to the type of carbohydrate, to the wheat gluten or to other factors. Effects of some other carbohydrates, of higher levels of wheat flour and of sorbitol in the diet were therefore studied. High doses of nicotinic acid reduced hypercholesteremia in human patients(3,4) and rabbits(5); so effects of high dietary levels of this vitamin and of feeding inositol-hexanicotinate† on serum cholesterol concentration in rats have also been studied.

*Methods.* Male weanling rats of Sprague-Dawley, Holtzman and Rolfsmeyer strains weighing 50-55 g were used. There were 6 rats in each group and the animals were fed *ad lib.* The basal diet contained hydrogenated coconut oil, 25%; cholesterol, 1%; cholic acid, 0.5%; salts, 4%; and casein, 25%, except in Exp. 1 (Table I) in which casein was fed at 40% level. All dietary changes were made at the expense of sucrose. Nicotinic acid and inositol-hexanicotinate were mixed with the diet. Vitamins were supplied in adequate amounts as described previously(6). At end of 3 weeks food was withdrawn for 15-18 hours, then blood was removed from each rat. Food consumption for each group was measured for entire experimental period. Serum cholesterol concentrations of individual rats were measured by the method of Henly(7). Oxidized pyridine nucleotides in blood(8) and both reduced(9) and oxidized(10) pyridine nucleotides in livers of animals ingesting high doses of nicotinic acid were estimated. Experimental procedures have been described (6).

*Results.* Substitution of either high extraction wheat flour or ground wheat for sucrose at a level of 29.5% in the diet, caused some reduction in serum cholesterol concentration (Table I, Exp. 1). When the carbohydrate was 44.5% of diet (Table I, Exp. 2), substitution of extracted wheat flour or ground wheat for sucrose again caused reduction in hypercholesteremia; the reduction with ground wheat being marked and significant. Dextrin (gelatinized corn starch), substituted for sucrose as the carbohydrate, did not affect serum cholesterol concentration. Substitution of 30% of lactose for sucrose in the diet, on the other hand, caused a more marked hypercholesteremia.

Since lactose is not likely to be consumed at such high levels, the effect of lower levels of lactose in the diet on serum cholesterol concentration was studied. The results are shown in Table I, Exp. 3. Diets containing 5-20% of lactose caused increases in serum cholesterol concentration; however, increasing the lactose level above 5% did not further elevate serum cholesterol concentration. Further evidence of hypercholesteremic effect of lactose was obtained when both lactose and wheat gluten were included in the diet. The fall in serum cholesterol concentration caused by supplemental amounts of wheat gluten was partially prevented by including 10% of lactose.

Changes in serum lipid phosphorus concen-

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† Inositol-hexanicotinate was kindly supplied by Philadelphia Ampoule Labs., Philadelphia, Pa.

TABLE I. Effects of Different Carbohydrates, Sorbitol, Nicotinic Acid and Inositol Hexanicotinate on the Serum Cholesterol Concentration in the Rat.

| | | | | Serum | | Liver | |
|---|---|---|---|---|---|---|---|
| Exp. No. | Dietary alterations | Avg daily food intake/rat, g | Avg wt gain (3 wk), g | Total cholesterol, mg % | Lipid P, mg % | Total lipids, % | Total chol., % |
| 1 | 29.5% sucrose | 9.7 | 109 ± 4* | 655 ± 76* | 5.8 | | |
| | 29.5% wheat flour (high extraction) | 9.4 | 109 ± 4 | 519 ± 61 | 5.5 | | |
| | 29.5% ground wheat | 10.5 | 125 ± 2 | 547 ± 46 | 6.4 | | |
| 2 | 44.5% sucrose | 8.8 | 98 ± 5 | 511 ± 64 | 5.4 | | |
| | 44.5% wheat flour (high extraction) | 11.2 | 133 ± 5 | 434 ±102 | 6.2 | | |
| | 44.5% ground wheat | 10.9 | 133 ± 5 | 326 ± 65 | 5.4 | | |
| | 44.5% dextrin | 9.9 | 110 ± 3 | 502 ± 79 | 6.1 | | |
| | 30% lactose + 14.5% sucrose | 8.1 | 85 ± 1 | 1050 ± 79 | 10.7 | | |
| 3 | Sucrose | 9.2 | 100 ± 5 | 539 ± 31 | 6.7 | | |
| | 5% lactose | 8.5 | 91 ± 3 | 687 ± 80 | 7.0 | | |
| | 10% " | 9.3 | 98 ± 4 | 747 ± 88 | 7.1 | | |
| | 15% " | 8.6 | 90 ± 3 | 695 ± 37 | 7.1 | | |
| | 20% " | 8.8 | 91 ± 3 | 665 ±121 | 7.3 | | |
| | 10% lactose + 10% gluten | 9.0 | 114 ± 4 | 408 ± 38 | 6.4 | | |
| | 10% wheat gluten | 9.8 | 114 ± 5 | 312 ± 30 | 4.9 | | |
| 4 | Sucrose | 9.7 | 95 ± 4 | 652 ± 58 | 7.7 | 16.5 | 6.51 |
| | 10% lactose (Tech) | 9.2 | 95 ± 3 | 733 ± 75 | 7.3 | 16.3 | 6.96 |
| | 20% " " | 8.7 | 92 ± 2 | 912 ± 86 | 9.1 | 19.0 | 7.22 |
| | 10% " (U.S.P.) | 9.5 | 94 ± 2 | 922 ± 62 | 9.1 | 16.7 | 7.09 |
| | 20% " " | 8.1 | 88 ± 2 | 1067 ± 62 | 10.9 | 17.7 | 7.67 |
| 5 | Sucrose | 8.7 | 109 ± 2 | 698 ± 60 | | | |
| | Glucose (A.C.S.) | 8.5 | 107 ± 3 | 652 ± 53 | | | |
| | " monohydrate | 9.9 | 111 ± 2 | 571 ± 58 | | | |
| 6 | 25% casein | 8.9 | 101 ± 3 | 539 ± 46 | 5.7 | | |
| | *Idem* + 10% sorbitol | 7.8 | 87 ± 3 | 804 ±101 | 8.2 | | |
| | " + .5% nicotinic acid | 8.6 | 97 ± 3 | 819 ±100 | 7.9 | | |
| | " + .57% inositol-hexanicotinate | 9.7 | 103 ± 2 | 655 ±123 | 7.2 | | |

* S. E. M.

tration paralleled changes in serum cholesterol concentration.

Failure to observe elevation of serum cholesterol concentration when lactose content was increased from 5% to 20%, and the observation that serum cholesterol concentrations of rats fed 30% of lactose in previous experiment were much higher (Table I, Exp. 2), suggested that source and purity of lactose might be the cause of the discrepancy. To test this, the effects of 2 grades of lactose, of sucrose and of glucose were compared.

Substitution of lactose for sucrose (Table I, Exp. 4) enhanced hypercholesteremia; however, the effect depended upon quality and purity as well as upon quantity of lactose in the diet. Ingestion of lactose also caused slightly higher deposition of cholesterol in the liver.

When sucrose was replaced with glucose in the diet (Table I, Exp. 5) there was no significant change in serum cholesterol concentration. However, feeding glucose-monohydrate‡ in place of sucrose caused a lowering of hypercholesteremia and a stimulation of food intake.

Table I, Exp. 6 shows that feeding 10% of sorbitol, 0.5% of nicotinic acid or 0.57% of inositol-hexanicotinate in the diet caused a rise in serum cholesterol concentration. Increasing the dietary level of nicotinic acid (Table II) also caused hypercholesteremia

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‡ Cerelose, Corn Products Refining Co., N. Y.

TABLE II. Effect of High Doses of Nicotinic Acid on the Lipid and Pyridine Nucleotide Concentrations in the Blood and Liver of Rats.

| Level of nicotinic acid in diet | Avg daily food intake, g | Avg wt gain (3 wk), g | Serum: Total chol., mg % | Serum: Lipid P, mg % | Blood PN, γ/ml | Liver: Total lipids, % | Liver: Total chol., % | Liver: PNH, γ/g | Liver: PN, γ/g |
|---|---|---|---|---|---|---|---|---|---|
| Control | 9.7 | 95 ± 4 | 652 ± 58* | 7.7 | 50 ± 1* | 16.5 | 6.51 | 278 | 644 |
| .5% N.A. | 8.7 | 88 ± 3 | 748 ± 48 | 7.2 | 71 ± 2 | 16.0 | 6.23 | 270 | 660 |
| 1.0% " | 9.0 | 90 ± 4 | 749 ± 111 | 7.4 | 76 ± 6 | 17.9 | 6.93 | 286 | 708 |
| 2.5% " | 8.5 | 83 ± 3 | 879 ± 59 | 7.4 | 73 ± 3 | 16.7 | 6.12 | 236 | 661 |

* S. E. M.

PN = Oxidized pyridine nucleotide. PNH = Reduced pyridine nucleotide.

under these conditions. Ingestion of high doses of nicotinic acid had no effect on liver lipids or on liver pyridine nucleotides. Oxidized pyridine nucleotides in blood, however, showed an appreciable and significant elevation over values for control animals consuming an adequate amount of nicotinic acid. No toxic effects of high doses of nicotinic acid were observed, except that animals receiving 2.5% level of nicotinic acid showed a slight growth depression.

*Discussion.* Reduction in serum cholesterol concentration when wheat flour was substituted for sucrose, is most likely due to cholesterol-lowering activity of wheat gluten(11). Failure of dextrin to reduce hypercholesteremia argues against an effect from the change in type of dietary carbohydrate.

Lowering of serum cholesterol values when starch is substituted for sucrose(1) may occur only when a severe hypercholesteremic regimen is employed. The effect of starch is attributed to its enhancement of cholic acid excretion but no such enhancement was observed by Matschiner *et al.*(12). Fillios *et al.*(13) using a more moderate dietary regimen found that cholesterol-lowering effect of starch disappeared after 3 weeks, while Adams *et al.* (14) found no cholesterol-lowering due to starch.

Cholesterol-lowering effect of glucose monohydrate is probably not the result of a change in kind of carbohydrate *per se.* It is more likely a result of stimulation of food intake and therefore of higher protein intake(11) or may be due to some impurity in this product. Substitution of anhydrous glucose for sucrose caused no such reduction (Exp. 5). Also a significant rise in serum cholesterol accompanying glucose feeding has been reported (14).

Higher liver and serum cholesterol values when lactose was fed, may be the result of increased intestinal absorption of cholesterol (15) or an effect of galactose on cholesterol metabolism. Sorbitol, reported to increase absorption of certain nutrients, also caused elevation in serum cholesterol concentration.

The different effects of different grades of a particular carbohydrate suggest that purity or moisture content of a carbohydrate may influence its effect on cholesteremia. This could explain plasma cholesterol-lowering observed with glucose monohydrate in chicks and rabbits(16).

The effect of high doses of nicotinic acid on serum cholesterol concentration in the rat differ from effects observed in man(3,4) and rabbits(5). Besides the possibility of species difference, nicotinic acid was administered as a single dose in experiments on man, whereas it was mixed in the diet in the present study. The suggestion(3) that serum cholesterol-lowering effect of high doses of nicotinic acid may be due to increased oxidative activity in the body leading to formation of "oxycholesterols," is not supported by the observation that feeding high doses of nicotinic acid caused increases in both oxidized pyridine nucleotide and cholesterol concentrations of blood. Merrill and Lamley-Stone(5) suggested that cholesterol-lowering effect of nicotinic acid may be due to its detoxication products. Schön(17) suggested that nicotinic acid depressed synthesis of cholesterol by bringing about a relative lack of coenzyme A. If this mechanism is operative, then little effect of nicotinic acid could be expected in our experiments since cholesterol feeding itself causes a marked decrease in cholesterol synthesis. Further, niacin deficiency does not have any effect on serum cholesterol concentration of mice fed a diet with or without added cholesterol(18).

*Summary.* When rats were fed a diet containing 25% of hydrogenated coconut oil, 1% of cholesterol and 0.5% cholic acid, substitution of wheat flour for sucrose caused a reduction in serum cholesterol concentration. Diets containing sucrose, dextrin and anhydrous glucose produced the same degree of hypercholesteremia. When sucrose was replaced in part with lactose or sorbitol hypercholesteremia was enhanced. Nicotinic acid at high levels and inositol-hexanicotinate at 0.57% level in the diet failed to lower serum cholesterol concentration. High doses of nicotinic acid had no effect on pyridine nucleotide content of the liver, but raised the oxidized pyridine nucleotide content of blood significantly.

Pyridine nucleotide determinations were kindly performed by Dr. Mary Morrison.

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# Studies on a Virus ("Polyoma") Inducing Multiple Tumors in Animals.* (25323)

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Induction of leukemia, parotid gland and mammary tumors, and subcutaneous sarcomas in mice by cell-free preparations of mouse leukemic organs was first reported by Gross (1,2). Stewart(3,4,5) induced by similar means in mice neoplasms of similar types and tumors of adrenal, submaxillary, sublingual and exorbital lacrimal glands. These observations have been confirmed by a number of workers(6). Gross(7) induced leukemia, parotid gland tumors and/or sarcomas with cell-free extracts of parotid gland neoplasms. Although Stewart, Eddy *et al.*(8) failed to obtain similar results, they induced with extracts of the same tumors or leukemic organs of mice, following passage on monkey kidney or on mouse (NIH-Swiss) embryo cultures, with exception of leukemia, a great variety of tumors in mice and hamsters(8-10). The name "polyoma" virus has been proposed for this agent which induces cytopathogenic changes in monkey kidney and mouse embryo cultures (11) and agglutinates red blood cells of guinea pig, hamster, rabbit, mouse and man(12). The virus produces both proliferative and degenerative changes(13) and infected animals develop hemagglutination inhibition(12) and complement fixing antibodies(14). The observations of Gross and Stewart have been confirmed and extended to other strains of mice and rats(15-18). The question arose whether a number or only one virus(19) is responsible for the multiplicity of tumors. Separation of leukemia-inducing virus from parotid tumor-inducing virus, first reported by Gross(2), has been confirmed(8,15). Leukemia in mice comprises a number of histological types(20), and at least some of them are induced by different viruses, as there is no immunological relationship between leukemia-inducing agent of Gross or of Schwartz(21) and that of Friend(22-23). The relationship of parotid gland tumor virus of Gross and the agent described by Stewart is not clear and requires elucidation. The present studies were undertaken to characterize any submicroscopic changes and specific structures which may be found in tissues infected with polyoma virus and in tumors induced by this agent and to compare them with those already reported in parotid gland tumors induced by cell-free preparations of leukemic organs from mice with leukemia of Gross(24-25).

*Materials and methods.* Several strains of polyoma virus were obtained through the courtesy of Dr. Sarah E. Stewart, as lyophilized tissue culture fluids. For tissue culture embryos of mice of the following strains were used, NIH (Swiss) supplied by Dr. Stewart, Af/Dm, C3Hf/An, C3Hf/Gr, and C3HZb/Bi. Minced embryos were treated for 20 minutes at room temperature with minimal volume of trypsin solution (0.25% Difco Bacto Trypsin in Ca and Mg free Hanks' B.S.S.), centrifuged, washed in B.S.S., resuspended in Eagle's basal medium containing 20% inactivated horse serum and dispensed in 2 ounce prescription bottles. After good growth was obtained, medium containing 2% horse serum was used as maintenance medium to which polyoma suspension was added. Fluids from cultures of embryos showing cytopathogenic changes were tested for hemagglutination of guinea pig red blood cells and for tumor-inducing activity by subcutaneous inoculation in newborn hamsters (0.2 ml doses) and in newborn NIH (Swiss) mice (0.1 ml doses). Cells of cultures showing cytopathogenic changes and tumors induced by culture fluids were studied in ultrathin sec-

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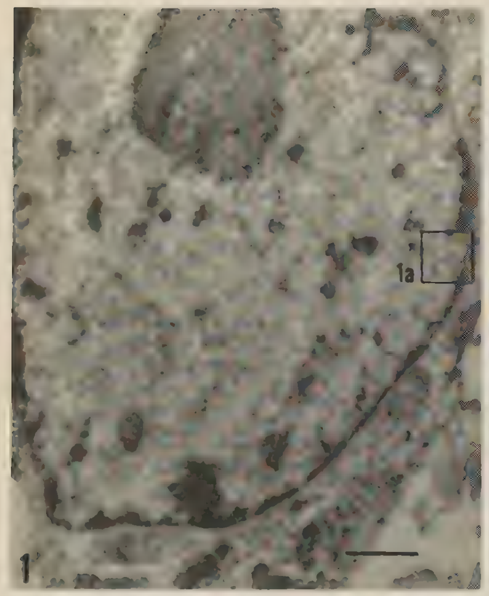

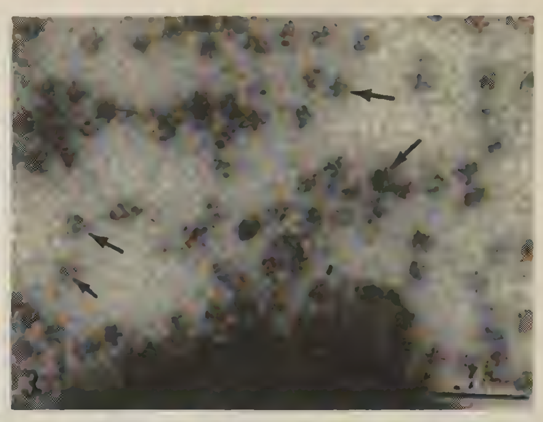

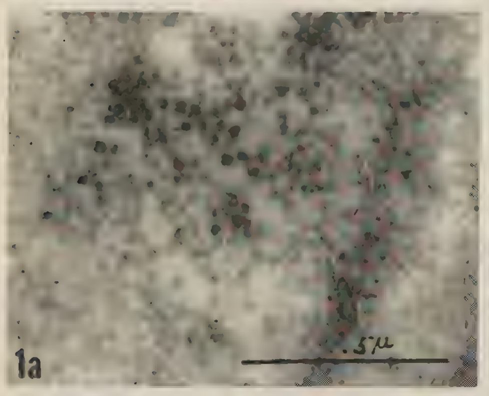

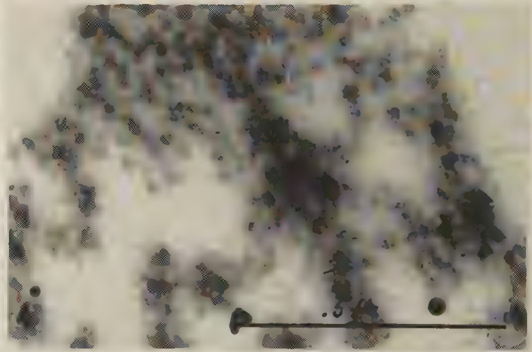

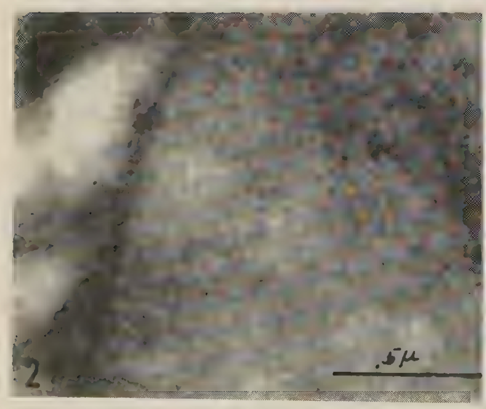

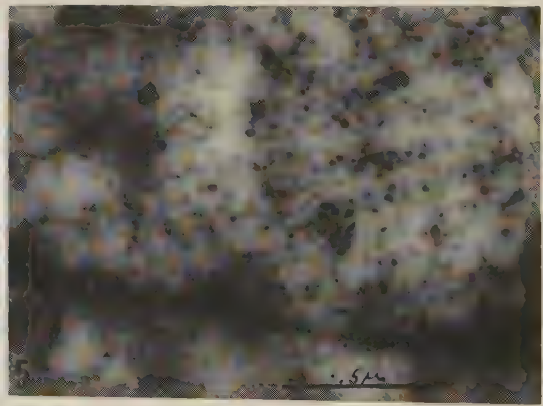

FIG. 1. Electron micrograph of section of a cell of Af strain mouse embryo grown *in vitro*, treated with polyoma virus. Particles scattered throughout nucleus and around nucleolus. × 10,000.

FIG. 1a. Part of Fig. 1 at higher magnification, showing characteristic particles near nuclear membrane. × 56,000.

FIG. 2. Part of nucleus of a cell from NIH (Swiss) mouse embryo culture, treated with polyoma virus, showing closely packed characteristic particles. × 40,000.

tions by means of RCA EMU-3E electron microscope. The method of tissue preparation for study in ultrathin sections was the same as previously described(24).

*Results.* Cytopathogenic changes, as described by Eddy *et al.*(11) have regularly been observed within 4 to 7 days in cultures of embryonic tissues of NIH (Swiss), Af/Dm, C3Hf/An, C3Hf/Gr, and C3HZb/Bi strain mice. Fluids from cultures of embryos from all strains of mice with cytopathogenic changes hemagglutinated guina pig red cells in variable titers of 1:80 to 1:2,560. Fluids from cultures of NIH (Swiss) and Af/Dm embryo mice, so far tested, induced within 2-3 months tumors in 5 out of 5 and 4 out of 4 surviving hamsters (sarcoma of the kidney and heart, lung adenocarcinoma, thymoma, hemorrhagic disease), and in aproximately 60% of surviving NIH (Swiss) mice, dwarfism, parotid gland and mammary tumors, and hemorrhagic disease.

An electron microscope study of sections of NIH (Swiss) and Af/Dm strain mouse embryo cells showing cytopathogenic changes revealed the presence of virus-like particles in many cells, chiefly in the nucleus, occasionally in the cytoplasm and outside the cells. The particles measure approximately 270 Å in diameter and are of low electron density. They may be scattered throughout nucleoplasm (Fig. 1 and 1a) or completely fill the nucleus (Fig. 2). At higher magnification, in some particles, an internal structure may be seen composed of a more dense center, measuring approximately 50 Å and surrounded by a less dense zone with outlines of a limiting membrane (Fig. 3, 3a, b, c, and d). Particles outside cells are of similar size and appearance (Fig. 4). Examination of cells grown *in vitro* from embryos of NIH and Af/Dm strain mice, untreated and maintained in tissue culture for similar intervals of time, failed to reveal any characteristic particles. Fluids from these cultures failed to hemagglutinate guinea pig red cells. In parotid gland(Fig. 5) and in mammary tumors induced by polyoma infected culture fluids, the nuclei and cytoplasm of cells showed characteristic particles observed in cells of infected tissue cultures. It is of interest that mammary tumors induced in NIH (Swiss) mice by polyoma virus have been found histologically different from mammary tumors induced by Bittner virus. The cells of these tumors progress from epithelial in character to fibroblast-like fusiform cells. The tumors contained only particles of 270 Å in size, but none of the larger size particles (900 Å) originally observed in mammary tumore of mice, known to harbor Bittner virus and developing, although at a much later age, a high incidence of mammary cancer(26).

*Discussion.* The parotid gland tumor agent of Gross and polyoma are known to share some physical properties, such as resistance to ether(2,7,12) and heating(2,12,27). Neutralization of the parotid gland tumor virus of Gross by sera of mice immunized against tissue culture preparations of polyoma(28) indicates antigenic similarity, but not necessarily identity of parotid gland tumor virus of Gross and of polyoma virus. The part played by normal mouse proteins in this neutralization effect still remains to be explored.

Electron microscope studies of leukemic organs of mice with spontaneous lymphocytic leukemia and of mice with similar type of leukemia induced by cell-free preparations of leukemic organs of mice revealed virus-like particles, similar in size and appearance (570 Å to 1650 Å), within and outside the cytoplasm of cells(29-30). In leukemia induced by cell-free filtrates of leukemic organs of mice with x-ray induced leukemia, the size of particles was smaller (400 Å to 670 Å)(31). In parotid gland tumors of mice induced by cell-free preparations of leukemic organs of mice, virus-like particles uniform in size and aver-

FIG. 3. Part of nucleus of cell from a mammary tumor induced in a NIH (Swiss) mouse with tissue culture fluid of NIH mouse embryo culture infected with polyoma. Some of the particles show internal structure. × 120,000.

FIG. 3a, b, c, d. Some of the particles at higher magnification. × 195,000.

FIG. 4. Extracellular particles in infected Af strain mouse embryo cells grown *in vitro.* × 72,000.

FIG. 5. Part of a cell from a parotid gland tumor induced in a NIH (Swiss) mouse with tissue culture fluid of NIH mouse embryo culture infected with polyoma. Group of particles adjacent to nuclear membrane. × 40,000.

aging 650 Å have been observed within and outside the cytoplasm of cells(24). Particles similar in size and appearance have also been found in high-speed centrifugal pellets of extracts of mouse leukemic organs which induced an incidence of 11% of parotid gland tumors in C3H/Gr strain mice(24-25). Similar particles have been observed, in confirmatory findings, in spontaneous(32) and induced leukemia in mice(33). The size of particles present in cells of a parotid gland tumor was not uniform and ranged from 750 Å to 1100 Å(33). Particles of similar size have also been demonstrated in other types of leukemia (34-35) and in plasmocytoma(36).

It appears of interest that electron microscope studies of parotid gland tumors induced in C3H strain mice by cell-free preparations of leukemic tissues from mice with spontaneous leukemia, failed to reveal the characteristic particles of 270 Å, chiefly observed in the nucleus of parotid gland tumors induced in NIH (Swiss) mice by fluids from polyoma infected NIH (Swiss) and Af/Dm embryo cultures. Thus, particles in parotid gland tumors, induced in mice of different strains by various means, differ not only in size but also in their relationship to various cell constituents, although these tumors are of similar histological appearance. While the characteristic particles have been observed in nuclei of numerous cells of mouse embryos grown *in vitro* and infected with polyoma, similar particles have only infrequently been found in nuclei of parotid gland and mammary tumor cells induced in mice by polyoma infected tissue culture fluids. It is not possible at this time to describe the frequency of occurrence of these particles in tumors induced by polyoma, as an extensive examination of tumor cells has not as yet been carried out. Recent electron microscope studies of tissue culture fluids from mouse embryo cells on which extracts of a parotid gland tumor and of a neoplastic mesenteric lymph node, both originally obtained from leukemic mice of Gross, have been passaged, showed in dried and shadowed with chromium preparations particles of 400 Å to 450 Å in diameter(37). Size of these particles in solution was 520 Å to 620 Å(37). Correlation of size of these particles with that of particles in sections of parotid gland tumors induced by extracts of Gross' leukemia(25) is difficult at this time. They do appear to be more closely related in size than with particles of 270 Å now reported in parotid gland and mammary tumors induced by polyoma. Further studies are required to correlate values of particles reported in this study with values of active particles reported in filtration of tissue culture fluids infected with polyoma through gradocol membranes(11). Should further studies demonstrate that the particles are indeed the agent responsible for multiple tumors, they would represent the smallest tumor-inducing virus so far observed.

*Summary.* Electron microscope examination of ultrathin sections of mouse (NIH-Swiss and Af/Dm strain) embryo cells grown *in vitro* and showing cytopathogenic changes following passage of polyoma virus preparations has been carried out. Spherical particles of 270 Å in diameter and of low electron density have been observed chiefly in the nucleus, also in the cytoplasm and outside the cells. In some particles an internal structure could be discerned, composed of a more dense center of 50 Å in diameter, surrounded by an electron translucent outer zone with outlines of a limiting membrane. Similar particles have also been found in parotid gland neoplasms and mammary tumors induced in NIH (Swiss) mice by polyoma infected tissue culture fluids. Particles of this type have not been observed in uninfected control cultures.‡

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‡ During preparation of this paper, the results of Banfield, W. G., Dawe, D. J., and Brindley, D. C., submitted to J. Nat. Cancer Inst., came to our notice. They have observed similar particles (270 Å) in cells of a murine lymphoma strain grown *in vitro* and treated with polyoma preparations. In addition, they observed particles similar in size to some of those observed by us in spontaneous and induced mouse leukemia.

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## Human Studies of Biologic Availability of Niacin in Coffee.* (25324)

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Teply *et al.*(1) found that roasted coffee contained about 10 mg niacin/100 g as measured biologically with *L. arabinosis* and later confirmed by rat assay(2). Niacin content increases with degree of roasting; values range from 7 to 46 mg/100 g(2). Coffee has not been considered as a source of niacin in the human diet. In view of its wide use as a beverage, it seemed desirable to determine the biologic availability of niacin in coffee for man. Niacin contributed by coffee might explain certain inconsistencies in epidemiologic studies relating dietary intake of niacin and

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TABLE I. Effect of Coffee on Excretion of Niacin Metabolites in 5 Patients.

| Mean daily excretion of niacin metabolites calculated as niacin (mg) | | Daily intake of niacin in coffee (mg) | % of added niacin excreted |
|---|---|---|---|
| Basal diet | Basal diet + coffee | | |
| 8.5 | 15.2 | 11.4 | 58 |
| 6.2 | 15.0 | 13.4 | 66 |
| 7.3 | 12.9 | 9.6 | 58 |
| 8.7 | 15.1 | 13.0 | 49 |
| 8.9 | 11.3 | 12.9 | 19 |

tryptophan to the incidence of pellagra.

*Materials and methods.* Five adult subjects, 3 males and 2 females, were placed on control diet which furnished approximately 10 mg niacin and 1000 mg tryptophan daily. After 10 to 14 days, coffee was added to the diet, 4 cups (150 cc each) daily for 10 days. Urinary excretion of niacin metabolites, $N^1$-Methylnicotinamide ($N^1$-Me) and the pyridone of $N^1$-Me (pyridone) were determined in consecutive 48 hour periods with slight modifications of published methods(3,4). Coffee was prepared in a standard manner in electric coffee maker, each cup of beverage representing 10 g coffee. Coffee beverage was analyzed microbiologically for niacin using *L. arabinosis* and was reasonably constant. Dark roast coffee was employed; one cup (150 cc) contained 2.4 to 3.4 mg niacin.

*Results.* Excretion of niacin metabolites became stabilized at a constant level after 4 days on control diet or a similar period on the diet with coffee added. Mean daily excretion of niacin metabolites ($N^1$-Me + pyridone) on the last 6 days of each dietary regimen are are given in Table I. The quantity of niacin furnished by coffee and the percent of this niacin excreted in urine as metabolites are also shown. A significant increase in excretion of niacin metabolites occurred in each subject when coffee was added to the diet amounting to 19 to 66%, average 50%, of niacin furnished by coffee. The small increase in subject 5 was due to his failure to consume some foods supplying niacin and tryptophan during period when coffee was administered. In our previous studies addition of 10 to 150 mg of niacinamide to a similar diet resulted in an average increase in excretion of niacin metabolites of 57% of administered dose. It seems evident that niacin in coffee is biologically available for man and that the vitamin is absorbed and excreted in a manner similar to that observed for pure niacinamide.

*Comments.* Minimal niacin requirement of adults, including niacin formed from tryptophan, is about 9-12 mg daily depending on body size and caloric intake(5). The dark roast coffee used furnished 9.6 to 13.4 mg of niacin daily, enough to meet this requirement. Light roast coffee contains less niacin, approximately 1 mg/cup(1) but could also contribute significant amounts of this vitamin to the diet. The amount of coffee consumed should be considered in estimating daily intake of niacin. Consumption of coffee in some countries in which staple foods are low in niacin and its precursor, tryptophan, may explain, in part, the infrequency with which pellagra is observed in these areas.

*Summary.* Coffee contains a significant amount of niacin which is biologically available for man. This beverage can furnish an appreciable share of daily requirement of niacin and should be considered in estimating the niacin content of the human diet.

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# Effect of Vit. E on Myoglobin Content of Guinea Pig Skeletal Muscle.* (25325)

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Increase in myoglobin concentration of guinea pig skeletal muscle during early stages of Vit. E-deficiency has been reported(1). This increase was postulated to be due to an increase in anabolic nature of myoglobin metabolism, principally because of the observation by Indovina(2) of an increase in myoglobin turnover in Vit. E-deficient rabbit skeletal muscle. During extended periods on Vit. E-deficient diet, there was a decrease in myoglobin content which Indovina attributed to increase in catabolism over anabolism. Indovina suggested a "normalizing" effect of Vit. E on myoglobin metabolism. A decrease in myoglobin content also has been noted in a) muscles of patients with progressive muscular dystrophy before onset of marked clinical disability(3), b) in calves with muscle degeneration(4). In genetically dystrophic mice diminished myoglobin concentration accompanied by creatinuria has been reported(5). Such observations can be interpreted as revealing either an actual defect in myoglobin metabolism or a passive, secondary loss from damaged muscles. The present study examined quantitatively the myoglobin changes in both gastrocnemius and masseter muscles from guinea pigs carefully selected for age, sex, and weight during prolonged Vit. E-deficient states. Furthermore, we examined the effect of Vit. E-supplementation on previously Vit. E-deficient animals, and correlated, where possible, all observations with other reported criteria of nutritionally induced dystrophy.

*Methods.* Forty-six English strain male guinea pigs, 30 days of age, were placed on Vit. E-deficient diet(6) for either 21 or 30 days. In the 21 day study, deficient, supplemented (control) and recovery animals were maintained through 14th day as previously described(1). Subsequently, the deficient animals were continued on the deficiency regimen, while the supplemented and recovery animals were given 15 mg of dl-alpha-tocopherol daily until the 21st day. Supplementation of the 30-day control animals with 15 mg of dl-alpha-tocopherol was performed twice weekly. On designated day of sacrifice the gastrocnemius and masseter muscles were removed and analyzed for myoglobin(1), total nitrogen, non-collagen-nitrogen (NCN) and percentage of solids(6). Muscle weight was expressed as percentage of total body weight. Daily creatine excretion was determined on 4 supplemented and 4 deficient animals throughout the 30-day period. Histological sections were made from samples taken randomly and equally from each dietary regimen and duration. Statistical analysis of data was by the small sample *t* test. A probability of $\leq 0.05$ was accepted as revealing a significant difference between the groups.

*Results.* No statistically significant difference between gastrocnemius or masseter muscles of deficient, supplemented or recovery animals in terms of percentage solids or weights (as percentage of body weight) is evident in the 21 day studies (Table I). Furthermore, no important alteration in water content or weight/body weight ratio is noticeable in muscles from guinea pigs maintained on Vit. E-deficient diet for 30 days. Non-collagen-nitrogen analyses reveal no significant changes except in the 21 day deficient gastrocnemii, which show a decline in NCN concentration. There is a significant diminution of myoglobin concentration (mg/g wet muscle) in deficient muscles at both dietary durations.

In Table II myoglobin concentrations are expressed in mg/g of non-collagen tissue. The percentage difference between control and experimental muscles at 21 and 30 days is not as great in the masseters as in the gastrocnemii; nevertheless, the difference is statistically significant in all instances.

Supplementation of previously deficient animals from 15th to 21st day (recovery study)

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* Aided by grant from Muscular Dystrophy Assn.

TABLE I. Means and Standard Errors of Myoglobin and Non-Collagen Nitrogen Concentration, Percentage Solids and Muscle Weight as Percentage of Body Weight of Gastrocnemius and Masseter Muscles of Guinea Pigs on Their Respective Diets and Durations.

| Dietary regimen and duration | | No. of cases | Myoglobin (mg/g wet muscle) | Non-collagen N (mg/g wet muscle) | % solids (dry wt/wet wt × 100) | Muscle wt/body wt × 100 |
|---|---|---|---|---|---|---|
| | | | Gastrocnemius | | | |
| 21 day | Def. | 13 | .964 ±.054* | 23.37 ± .92* | 23.64 ±.24 | .497 ±.016 |
| | Suppl. | 10 | 1.373 ±.085 | 26.29 ± .69 | 24.03 ±.45 | .501 ±.018 |
| | Rec. | 10 | 1.240 ±.089† | 26.10 ± .69† | 23.68 ±.33 | .499 ±.011 |
| 30 day | Def. | 7 | 1.141 ±.057* | 23.75 ± .34 | 23.65 ±.45 | .478 ±.026 |
| | Suppl. | 6 | 1.519 ±.073 | 25.31 ± .64 | 23.88 ±.49 | .503 ±.024 |
| | | | Masseter | | | |
| 21 day | Def. | 13 | 3.128 ±.085* | 24.31 ± .54 | 23.89 ±.23 | .718 ±.026 |
| | Suppl. | 10 | 3.740 ±.167 | 26.73 ±1.08 | 23.58 ±.42 | .715 ±.024 |
| | Rec. | 10 | 3.665 ±.223† | 25.43 ± .54 | 24.02 ±.30 | .735 ±.020 |
| 30 day | Def. | 7 | 3.136 ±.049* | 24.01 ± .56 | 23.89 ±.35 | .727 ±.033 |
| | Suppl. | 6 | 3.926 ±.197 | 25.16 ± .43 | 24.16 ±.36 | .704 ±.021 |

* Probability of difference between deficient and supplemented groups ≤ .01.
† " " " " " " recovery groups ≤ .01.

prevents depletion of myoglobin and maintains the NCN level. No elevation in urinary creatine appears even after 30 days on the deficient diet. The histological picture of gastrocnemius muscles in the 21 day deficient group is one of mild or incipient degenerative changes; this state is not evident in either the recovery or supplemented groups. At 30 days, the pathology in deficient gastrocnemii approaches that described by West and Mason (7) as a sub-lethal type of injury. In contrast, masseter muscles from both 21 and 30 day deficient groups exhibit only rare and very mild cytological alterations.

TABLE II. Mean and Standard Error, Percentage Difference and Probability of Difference in Myoglobin Concentration per Gram of Non-Collagen Tissue.

| Dietary regimen and duration | | Myoglobin (mg/g NCN tissue) | % difference (Def. vs suppl.) | Probability (Def. vs suppl.) |
|---|---|---|---|---|
| | | Gastrocnemius | | |
| 15 day | Def. | 1.844 ±.125 | | |
| | Suppl. | 1.508 ±.055 | +22.3 | .030 |
| 21 day | Def. | 1.291 ±.078 | | |
| | Suppl. | 1.687 ±.100 | −23.5 | .004 |
| | Rec. | 1.586 ±.110 | | |
| 30 day | Def. | 1.520 ±.089 | | |
| | Suppl. | 1.912 ±.083 | −20.8 | .008 |
| | | Masseter | | |
| 15 day | Def. | 4.725 ±.206 | | |
| | Suppl. | 3.738 ±.248 | +26.3 | .034 |
| 21 day | Def. | 4.114 ±.140 | | |
| | Suppl. | 4.640 ±.290 | −11.4 | .040 |
| | Rec. | 4.605 ±.295 | | |
| 30 day | Def. | 4.087 ±.118 | | |
| | Suppl. | 4.901 ±.204 | −16.4 | .006 |

*Discussion.* Short-term lack of Vit. E in the diet produces a progressive and marked increase in myoglobin concentration(1). Extension of the deficiency duration precipitates an equally marked decline in the concentration. These changes in muscle pigment occur in both red (masseter) and pale (gastrocnemius) muscles before appearance of severe cytological lesions or of elevated creatine excretion. It is appropriate, however, to consider 2 sources of error in estimating the myoglobin status. First, there is the possibility of differences in dilution resulting from inequalities in muscle water content. This aspect has been remarked upon by Anthony *et al.*(8). Second, an analogous situation might arise if there were significant changes in the non-collagen/collagen tissue ratio.

Although there was no evidence of a significant change in water content, in view of the observed decline in NCN of the 21 day deficient gastrocnemii, all myoglobin data were recalculated and expressed (Table II) as mg/g non-collagen tissue. The re-evaluation yielded an identical conclusion: *i.e.*, that depression in myoglobin concentration was real and of reasonably great magnitude. Fifteen-day values were included to emphasize how suddenly the concentration was reversed, from 22% greater to 23% less than control levels in the case of gastrocnemii. It is apparent that the percentage decline was considerably smaller in the red muscle than in the pale muscle. This finding is in accord with the statement by Aloisi(9) that the pale muscles of the guinea pig are more sensitive to Vit. E-deficiency than are red muscles. Moreover, Goettsch and Pappenheimer(10) have reported that the masseter is the last muscle in the guinea pig to reveal any pathological lesion of nutritional dystrophy.

Increase in myoglobin during early stages of Vit. E-deficiency is probably due to an anabolic error. The subsequent decrease in myoglobin concentration after 21 days is subject to one or both of 2 interpretations: a) catabolism exceeds anabolism; or b) passive loss from muscle occurs. Certainly the decline in myoglobin concentration is greater than can be accounted for on the basis of degenerative changes observed in histological sections. On the other hand, even the presence of rare and mild cytological lesions might imply subtle and extensive membrane permeability changes not recognizable in histological sections. That an abnormal muscle permeability occurs in mice with hereditary muscular dystrophy has been suggested by the studies of Zierler(11).

Supplementation of previously Vit. E-deficient animals, as performed in 21 day recovery studies, prevents the abrupt and marked reversal in myoglobin concentration observed in the 21 day deficient animals. Although there is an actual decline in pigment concentration in recovery guinea pigs, the magnitude of concentration at 21 days is comparable to that recorded for control animals. The response of recovery animals to Vit. E-supplementation attests to the immediacy and potency of the vitamin in "normalization" of myoglobin metabolism.

*Summary*. A marked decline in myoglobin concentration has been demonstrated in gastrocnemius and masseter muscles of guinea pigs maintained longer than 15 days on a Vit. E-deficient diet. Supplementation with Vit. E after 14 days on a deficient diet reduces the magnitude of concentration change and, indeed, stabilizes pigment concentration at control levels. No significant difference between control and experimental animals in regard to water content, muscle weight or body weight was evident from 21 and 30 day dietary regimen studies. Non-collagen nitrogen was significantly depleted only in the gastrocnemii of the 21 day deficient animals.

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# Turbidity Produced by Hexamminecobaltic Chloride in Serum of Rabbits Injected Intravenously with Papain.* (25326)

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Collapse of normally rigid ears of young rabbits can be produced by intravenous injection of crude papain(1). This phenomenon is associated with loss of chondroitin sulfate from the matrix of cartilage throughout the animal(2,3). Similar changes occur in cartilage following injection of crystalline papain protease inactivated prior to injection(4). In previous studies, sulfur$^{35}$ technics and the carbazole method(2) were used to demonstrate marked increases in level of serum chondroitin sulfate following injection of crude papain by vein. The purpose of this paper is to describe a new and simpler method for determination of sulfated mucopolysaccharides in serum, and to present data obtained by this method in the course of recent *in vivo* experiments with crystalline papain protease. The trivalent hexamminecobaltic cation ($Co(NH_3)_6^{+++}$) precipitates chondroitin sulfate and its protein complex from dilute aqueous solutions and from serum(5). While normal serum diluted 1:10 gave no increased turbidity with hexamminecobaltic chloride, serum from rabbits injected with appropriate preparations of papain regularly showed increased turbidity following addition of the cation. This effect was enhanced by preliminary dialysis of the serum. This reaction was further studied in the following experiments.

*Materials and methods.* Hexamminecobaltic chloride and potassium chondroitin sulfate were prepared as described previously(5). Crude papain was prepared for injection as described by Thomas(1), while twice recrystallized papain protease was prepared by the method of Kimmel and Smith(6) from dried papaya latex‡ or obtained from Nutritional Biochemicals, Inc. The crystalline protease was inactivated with regard to its action on synthetic substrate benzoyl-l-arganinamide either by dialysis or by treatment with iodoacetamide as described by McCluskey and Thomas(4). In most experiments, the excess iodoacetamide was removed by dialysis following reaction with papain. Young rabbits weighing approximately 1 kg were injected by vein with 1 ml of various papain preparations, and blood drawn by cardiac puncture at intervals noted below. Control animals were handled in like fashion, but not injected with the enzyme. Sera were analysed turbidimetrically by one of 2 methods. *Procedure A*: 1.0 ml sample of serum was dialysed against 6 liters of distilled water at 4°C for 48 hours, and final volume of sample recorded. 0.2 ml aliquot was placed in 10 X 75 mm Coleman Jr. spectrophotometric cuvette, mixed with 2 ml of distilled water, and apparent absorbance at 615 mμ was recorded. Then 0.2 ml of a 0.5% w/v aqueous solution of hexamminecobaltic chloride was added, and the tubes inverted thrice. After 5 minutes turbidity was measured as the apparent increase in absorbance at 615 mμ (Δ O.D. in the Tables) and corrected for volume increase during dialysis. *Procedure B*: In similar fashion, 0.4 ml of serum was dialysed 48 hours at 4°C, then centrifuged at 2,000 rpm for 15 minutes. The small protein precipitate was discarded and the supernatant transferred to cuvettes calibrated at 2.2 ml. After addition of distilled water to that mark, absorbance was measured at 615 mμ, the cobalt reagent added as above, and the absorbance increment again recorded. The pH of dialysed serum was approximately 7.0.

*Results.* Using Procedure A, turbidities were determined for several experimental groups at times indicated in Table I. Potassium chondroitin sulfate, 150 mg/kg or the

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‡ Provided by Wallerstein Labs., N. Y.

TABLE I. Mean Absorbance Increments in Serum of Rabbits following Papain or Chondroitin Sulfate Intravenously (Procedure A).

| Material inj. | Time | | | | | No. animals |
|---|---|---|---|---|---|---|
| | 0 hr | 5 min. | 6-8 hr | 24 hr | 72 hr | |
| Chondroitin sulfate, 150 mg/kg | .001 | .154 | .060 | .000 | .000 | 2 |
| Crude papain, 1 ml of 1% solution, 10 mg/kg | .015 | | .164 | .150 | .067 | 11 |
| Crystalline papain protease treated with iodoacetamide, 5 mg/kg | .003 | | .070 | .258 | .025 | 12 |
| Crystalline papain protease + 30 mM cysteine, 5 mg/kg | .018 | | .030 | .053 | .032 | 5 |
| Controls | .015 | .002 | .007 | .019 | .028 | 11 |

various papain preparations were injected at time 0. Peak plasma levels occurred 5 minutes after injection of chondroitin sulfate, with complete disappearance at 24 hours. Of the several papain preparations, only the cysteine-activated crystalline enzyme failed to produce significant turbidities. Papain dialysed to remove cysteine produced turbidities similar to those recorded for iodoacetamide-treated papain. Detailed analysis of these and similar experiments will be reported.

The material precipitated was next studied by administration of sulfur$^{35}$. Over 80% of this isotope is excreted in the first 24 hours following injection, the rest being rapidly incorporated into chondroitin sulfate as well as other sulfur-containing compounds(7). Two rabbits were each given 0.5 millicurie of carrier-free $S^{35}$ as $Na_2S^{35}O_4$ intraperitoneally, and 5 days later one of them was given one ml of crude papain solution (10 mg/kg). Samples of serum processed by Procedure B were analysed for radioactivity. Then 0.1 ml aliquots of whole serum, dialysed serum, and dialysed serum after precipitation of chondroitin sulfate by hexamminecobaltic chloride were placed in 2.5 cm planchettes and oven-dried at 90°C for at least 48 hours. These were counted in duplicate in a gas-flow counter with a micromil end-widow (Nuclear, Chicago). Table II gives the result for papain-treated and control rabbits, both having similar counts/minute in whole serum initially. Both were bled 24 hours following injection of enzyme. It is apparent that most of the non-dialysable $S^{35}$ activity is precipitable by the cation, and this is in keeping with the increase in absorbance. Not only was the amount of $S^{35}$ precipitable by the cation proportional to the increase in non-dialysable $S^{35}$, but it was closely related to the apparent increase in absorbance at 48 and at 72 hours ($\Delta$ O.D.). At this latter time, there was no longer any difference between the papain-treated and control animal.

TABLE II. Comparison between $S^{35}$ Activity (Counts/Minute, cpm) in Serum Fractions, and Absorbance Increment ($\Delta$O.D.) following Hexamminecobaltic Chloride, in Control and Crude Papain-Treated Animals (Procedure B).

| | Control | Papain |
|---|---|---|
| Before papain (time 10 hr) | | |
| Initial whole serum cpm | 14,000<br>(± 1,000) | 16,000<br>(± 1,000) |
| Initial whole serum $\Delta$O.D. | .039 | .010 |
| After papain (time 24 hr) | | |
| Whole serum cpm | 10,617 | 37,447 |
| Dialyzed serum cpm | 8,547 | 38,264 |
| Dialyzed serum $\Delta$O.D. | .044 | .216 |
| Dialyzed serum after removal of cobalt ppt. cpm | 8,985 | 12,924 |
| Cobalt ppt. (by subtraction) cpm | −438 | +25,340 |

At concentrations ranging between 0.1 and 1.0 mg/ml, several substances, mostly polyanions, precipitated *in vitro* with the test reagent. These are heparin (Liquaemin Roche), heparitin sulfate (Upjohn), thymus desoxyribosenucleic acid, ribosenucleic acid, thymus desoxyribosenucleic acid-protein complex,§ aortic protein polysaccharide complex, aortic polysaccharide. Substances that did *not* precipitate with hexamminecobaltic chloride at these concentrations in dilute aqueous solutions were: human serum albumen, human serum globulin (Fraction II), serum mucoprotein prepared by the method of Winzler, umbilical cord hyaluronate, or any of the papain preparations. No desoxyribosenucleic acid was present in serum of papain treated animals at either 8 or 24 hours as determined by diphenylamine reactions[||] nor were there, at 8 hours, changes discernible by paper electrophoresis of test sera.

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§ By courtesy of Dr. Norman Cooper

To identify the precipitated material as chondroitin sulfate, several sera showing turbidity after addition of the cobalt reagent were examined further. Prior incubation of such sera with 75 TRU of testicular hyaluronidase (Wydase, Wyeth) at pH 4.5 for 48 hours, abolished the increment in apparent absorbance following dialysis and addition of the cobalt reagent. One ml samples of such sera were treated with an equal volume of 10% trichloracetic acid, the protein removed by centrifugation, and the supernatant dialysed against distilled water for 48 hours at 4°C. After addition of 0.4 ml of hexamminecobaltic chloride, the resultant precipitates were separated and dissolved in about 1 ml of 1% potassium acetate. The solutions were stirred 2 hours with approximately 500 mg of potassium Dowex-50 in 10 ml of distilled water, and the resultant solutions spotted on Whatman #1 paper. Toluidine blue staining of such a spot gave a metachromatic color, while associated protein was shown in other spots by staining with bromphenol blue. Another spot was chromatographed for 17 hours in 50% aqueous ethanol containing 0.1 M phosphate buffer at pH 7.0. The $R_f$ of this material matched that of known chondroitin sulfate in this solvent.

Aqueous solutions of potassium chondroitin sulfate were prepared at concentrations from 25 to 2,500 mg/ml. One ml aliquots of these were added to (a) 1.2 ml of distilled water, (b) 0.2 ml of dialysed rabbit serum and 1.0 ml of distilled water, and (c) 0.4 ml of dialysed rabbit serum and 0.8 ml of distilled water. The increase in turbidity (Δ O.D.) following addition of 0.2 ml of hexamminecobaltic chloride in each of these series bore a reproducible, essentially linear, relationship to chondroitin sulfate added, as shown in Fig. 1. Serum itself produced some increase of turbidity at low concentrations (0 to 0.5 mg/ml) of chondroitin sulfate whereas at higher concentrations (0.5 to 2.0 mg/ml) turbidities were depressed below those in water probably due to the ionic effects of protein. Using curves (b) and (c) as standard curves, there appeared to be 50-fold increase of serum chondroitin sulfate-like material following injection of biologically active papain in rabbits. This is in agreement with the range of values found by the carbazole method(2).

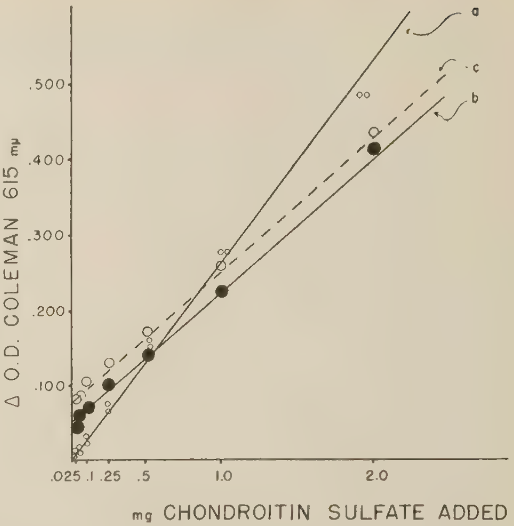

FIG. 1. Turbidities or absorbance increments (ΔO.D.) produced by hexamminecobaltic chloride and chondroitin sulfate in various media. Curve a: 1 ml chondroitin sulfate solutions, 1.2 ml distilled water, .2 ml cobalt sol. Curve b: 1 ml chondroitin sulfate solutions, 1 ml distilled water, .2 ml cobalt sol., .2 ml dialyzed rabbit serum. Curve c: 1 ml chondroitin sulfate sol., .8 ml distilled water, .2 ml cobalt sol., .4 ml dialyzed rabbit serum.

*Discussion.* The foregoing method is convenient for measuring chondroitin sulfate levels of serum in amounts (250 to 3,500 μg/ml) appearing in animals given papain. Due to its relative simplicity, many samples may be processed at once. However, because of the minute amounts of sulfated mucopolysaccharide found in normal rabbit or human serum (maximally 3 μg/ml) there is little likelihood of this test being clinically useful, except in conditions where there is a tenfold or greater increase in sulfated mucopolysaccharide. Coprecipitation of protein seems to occur, since the redissolved precipitate which contains chondroitin sulfate gives color as well with protein stains. That some of the protein

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[||] Performed by Dr. John Ayvazian.

moiety of chondromucoprotein remains attached to chondroitin sulfate is another possibility. As by the carbazole method for hexuronate, experiments with chondroitin sulfate in the range 250 to 1,000 $\mu$g/ml yield recoveries of 60 to 100% in the presence of protein. A similar result using the method of Bollet *et al.*(8) has been reported by Castor(9) for hyaluronate. This variation makes it possible to speak only of chondroitin sulfate-like equivalents in evaluating results of these determinations.

Since dialysis against 1/150 M phosphate buffer at pH 7.0 interfered with turbidity produced by $Co(NH_3)_6^{+++}$, distilled water was used in these studies. Euglobulins, however, precipitate with chondroitin sulfate at low pH in aqueous solution, therefore it was important to rule this out. Euglobulin precipitates under test conditions contained no appreciable amount of $S^{35}$ activity, and indeed were minimal.

During these experiments, it was found that if whole serum of rabbits given chondroitin sulfate alone, or treated with papain, were diluted 1:60 with 0.01 M acetic acid/acetate buffer at pH 4.48, turbidities proportional to amount of chondroitin sulfate present were obtained. This is similar to the "acid-precipitable globulin" method described by Greenspan(10) and may explain his findings of increased turbidity in human heparinised plasma as opposed to plasma oxalated and diluted in like manner. As this latter test was dependent upon secondary protein changes in serum, it was not employed quantitatively. Similarly, cetylpyridinium chloride turbidity is vitiated by such protein changes.

*Summary.* 1. Serum of rabbits following injection of chondroitin sulfate, crude papain, or several modifications of papain, became turbid following addition of hexamminecobaltic trivalent cation. Turbidity was directly related to amount of chondroitin sulfate-like material present in the serum. 2. The precipitate produced by this cation contained most of the non-dialysable $S^{35}$ activity present in the circulation following injection of crude papain, was metachromatic when redissolved, and did not form after incubation of serum with testicular hyaluronidase. 3. Addition of hexamminecobaltic chloride to dilute, dialysed serum is a useful way of demonstrating sulfated mucopolysaccharides in serum at levels exceeding 250 $\mu$g/ml.

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# Sulfated Mucopolysaccharides of Urine and Organs in Gargoylism (Hurler's Syndrome) II. Additional Studies.* (25327)

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In gargoylism or Hurler's Syndrome, 2 characteristic features have been reported: (1) the intracellular storage of one or more sulfated polysaccharides(1,2,3,4), and (2) excessive excretion in urine of 2 distinct muco-

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polysaccharides, chondroitin sulfate B and heparitin sulfate, neither of which is found in detectable amounts in normal urine(3,4). In this paper, data are reported on mucopolysaccharide excretion in urine and on the content of various organs obtained at autopsy of additional cases. It is concluded from these data (1) that various organs contain or store both mucopolysaccharides, and (2) that the ratio of these mucopolysaccharides differs in various organs from the same individual.

*Methods.* Mucopolysaccharides were precipitated from urine at pH of approximately 5 to 6 by addition of an aqueous 5% solution of cetyltrimethyl ammonium bromide or by cetylpyridium chloride as described previously(4). Usually, after purification, only 2 fractions were collected, the Ca salts precipitated at an alcohol concentration of 25% and of 50%. The first fraction consisted mostly of ChS-B and the second of heparitin sulfate although each fraction was contaminated to some extent with the other mucopolysaccharide. The relative concentrations of ChS-B and of heparitin sulfate were calculated from ratios of carbazole and orcinole and from their relative content of galactosamine and glucosamine, respectively. In many instances, an additional criterion of relative concentrations was utilized by determining degree of hydrolysis of each fraction after incubation with a flavobacterium extract adapted to ChS-B, followed by further incubation with heparitin-sulfate adapted enzyme(5). The organs available for study were kept frozen and shipped in dry ice. Specimens for analysis were ground in a tissue grinder, dehydrated in acetone, and defatted with ether. The material was then filtered, washed with acetone and ether, and dried *in vacuo*. Mucopolysaccharides were isolated from tissues after digestion with pepsin at pH 1.5, followed by tryptic digestion at pH 7.5 in the presence of toluene. Further purification and fractionation of the Ca salts was carried out as described previously(6).

*Results.* Urinary concentration of mucopolysaccharides in additional cases of gargoylism is listed in Table I. This table also lists concentration of mucopolysaccharides of 3 patients who did not have gargoylism. Since urine volume is normal, it can be seen that all

TABLE I. Chondroitin Sulfate B and Heparitin Sulfate of Urine in Gargoylism.

| Patient | Sex | Age, yr | Urine conc.: Total quantity (mg/l) | ChS-B (%) | Heparitin sulfate (%) | Remarks |
|---|---|---|---|---|---|---|
| 1 | ♂ | 8 | 85 | 58 | 42 | |
| 2 | ♂ | 20 | 58 | 74 | 26 | Siblings with all symptoms of the disease, mentally both normal (Dr. L. Abt., Phila.) |
| 3 | ♀ | 18 | 78 | 78 | 22 | |
| 4 | ♂ | 4.5 | 74 | 66 | 34 | |
| 5* | ♂ | 25 | 154 | 61 | 39 | Sex-linked recessive form, normal I.Q. |
| 6† | ♂ | 10.8 | 33 | ∼50 | ∼50 | |
| 7 | ♀ | 3.9 | 74 | ∼90 | ∼10 | |
| 8 | ♂ | 2 | 194 | 70 | 30 | |
| 9 | ♀ | 1 | 132 | 72 | 28 | |
| 10 | ♂ | 7.5 | 245 | 62 | 38 | 5 days prior to medication, creatinine 588 mg/l |
| | | | 179 | 64 | 36 | 5 days on prednisone (30 mg/day), creatinine 515 mg/l |
| | | | 155 | 71 | 29 | 11 days on prednisone (30 mg/day), creatinine 582 mg/l |
| 11 | ♂ | 9 | 9 | | | All Ch-S, no B, predominantly A, achondroplasia |
| 12 | ♀ | 7 | 16 | | | *Idem* |
| 13 | ♀ | 1.2 | 4 | | | Probably not gargoylism |

* Obtained from Dr. G. A. Jervis, Letchworth Village, N. Y.

† This patient of Dr. McKusick, Baltimore, Md., was markedly retarded in his mental development and had skeletal changes consistent with mild Hurler's Syndrome. He had no hepatosplenomegaly and no limitation of motion.

TABLE II. Chondroitin Sulfate B and Heparitin Sulfate Fractions in Gargoylism.

| Patient | Sex | Age, yr | Organ | Total quantity | ChS-B | Heparitin sulfate | Remarks |
|---|---|---|---|---|---|---|---|
| | | | | (%) | | | |
| 1* | ♂ | 12 | Liver | 6.7 | 6 | >90 | |
| | | | Spleen | 2.4 | 65 | ∼25 | |
| | | | Brain | .7 | 40 | 60 | |
| | | | Kidney | 1.5 | 50 | ∼50 | |
| 2† | ♂ | 10 | Liver | 1.2 | 56 | 44 | |
| 3‡ | ♂ | 7 | Liver | 6.3 | 5 | ∼90 | |
| | | | Brain | .8 | ∼40 | ∼30 | |
| 4§ | ♂ | 10 | Liver | 2.5 | 6 | 94 | |
| 5‡ | ♀ | 12 | Brain | 1.8 | 85 | 15 | |
| 6‖ | ♂ | .6 | Brain | .3 | | ∼40 | Normal brain; 60% hyaluronate, no ChS |

* Obtained from Dr. J. H. Austin, Portland, Oreg. † Obtained from Dr. W. A. Reiley, San Francisco, Calif. ‡ Obtained from Dr. G. W. F. Edgar, Amsterdam, Holland. § Obtained from Dr. G. A. Jervis, Letchworth Village, N. Y. ‖ Obtained from Dr. W. A. Blanc, Babies Hospital, N. Y. City.

cases of gargoylism had an increased excretion of total mucopolysaccharide and that ChS-B was the predominant mucopolysaccharide excreted in majority of instances.

Cases #2 and #3 are of especial interest. These two siblings had advanced and typical clinical symptoms of the disease including cloudy corneas but were of normal mental development. The similar ratio of ChS-B to heparitin sulfate in these siblings is notable. In Case #10, prednisone therapy (30 mg/day) was instituted for 11 days after a control period of 5 days. No appreciable change in creatinine excretion occurred during this period. Following institution of prednisone, a significant decrease in total mucopolysaccharide excretion occurred; however, the ratio of ChS-B to heparitin sulfate was not significantly altered.

Cases #11, 12 and 13 may be regarded as control subjects. Although Case #12 may have an increased polysaccharide excretion, no ChS-B or heparitin sulfate was detected. Infrared spectroscopy showed the fraction to be mainly ChS-A.

The organs of patients with gargoylism investigated included liver, spleen, brain and kidney. The results are listed in Table II.

As seen from this Table all organs of cases of gargoylism contained varying amounts of both ChS-B and heparitin sulfate. In Case #1, a 12 year old boy, the liver contained 6.7% (fat-free dry weight) polysaccharide; the latter consisted of about 90% heparitin sulfate. However, even in this case, a small fraction obtained at low alcohol concentration was identified as ChS-B. It seems remarkable that the spleen of this case contained 65% of ChS-B and only approximately 25% of heparitin sulfate, the rest being composed of other mucopolysaccharides. The liver in Cases #3 and #4 had a mucopolysaccharide partition very similar to that of Case #1 although the concentration in Case #4 was considerably lower. The liver of Case #2, however, had a ChS-B content equal to or higher than that of heparitin sulfate. The brains, including meninges, in Cases #1 and #3 had a low total mucopolysaccharide content while in Case #5, it was increased. It may be significant that this case had a high ChS-B content.

Since no comparisons with normal brain values were available, one brain including meninges of a child with no obvious pathology of the central nervous system, was analysed (Case #6). The mucopolysaccharide content of this brain was lower than any of the cases of gargoylism. On fractionation, no ChS was encountered. The only mucopolysaccharides which could be identified consisted of hyaluronic acid and heparitin sulfate.

*Discussion.* Our data are similar to those reported by Dorfman and Lorincz(3) and from this laboratory, in urine and liver, in liver by Brown(2), and in liver and spleen by Stacey and Barker(7). The last two laboratories apparently isolated from liver and spleen fractions corresponding to heparitin sulfate which apparently, in the majority of cases, occurs in the liver almost to the exclusion of other polysaccharides. It appears most noteworthy that the same individual may contain ChS-B as the major component of some organs and heparitin sulfate as the major component of others.

In all organs investigated from patients with Hurler's syndrome the concentrations of polysaccharides were greater, in most cases excessively so, than those of control organs. In unpublished experiments with organs such as liver, spleen, and kidney, the concentration of polysaccharides did not exceed 0.2% of the defatted dry weight. Amounts of polysaccharides isolated in most cases were insufficient to allow adequate characterization.

It is not known which cells produce and which ingest the polysaccharides from the extracellular fluids. Probably in the normal, some type of differentiated fibroblasts produces ChS-B and some cells of blood vessel walls produce heparitin sulfate. Experimentally, normal human macrophages in tissue culture have been shown by Mr. Morris and Dr. Godman of this school‡ to take up added heparitin sulfate from the culture medium and store it in the protoplasm in the form of discrete metachromatic granules similar in size to those of eosinophil granules. In Hurler's syndrome, polysaccharides contained in some parenchymatous cells may originate through a similar process of uptake from the circulating fluids. What factors determine the preferential storage of one or the other polysaccharide and the sparing of some organs (brain, for example) is not known.

The primary genetic defect of gargoylism remains obscure. As in other storage diseases, the intracellular storage is a gradually increasing process which manifests itself clinically later than the increased urinary excretion(4). The large quantities of polysaccharide excreted daily in these individuals strongly indicates an overproduction of the 2 polysaccharides. At present, our previous hypothesis, namely that the defect may be in a faulty differentiation of fibroblasts, appears still most plausible. According to this hypothesis, the connective tissue cells producing ChS-B and heparitin sulfate are increased possibly at the cost of those producing other mucopolysaccharides. One possible site of this defect is the blood vessel wall where ChS-B and heparatin sulfate occur normally together in relatively high concentration(8).

*Summary.* 1) Further examples of quantity and types of urinary excretion of sulfated mucopolysaccharides in Hurler's syndrome have been studied in 9 cases. All excreted both ChS-B as the major component and heparitin sulfate as a minor constituent. 2) In one case, excretion of mucopolysaccharides dropped after prednisone administration. 3) From various organs of 5 autopsy cases, the 2 polysaccharides were isolated. 4) Livers of 4 cases contained between 1.2 and 6.7% of total mucopolysaccharides (on fat-free dry wt). In 3 of these, 90% was heparitin sulfate, but from all of these, also some ChS-B was obtained. One liver had approximately equal quantities of the 2 polysaccharides. 5) In one case, spleen, brain and kidney of the same individual also yielded both polysaccharides, but in proportions remarkably different from those of liver. Brain of 2 additional cases also yielded the 2 mucopolysaccharides. One of these contained 85% of ChS-B. The brain of a normal child did not yield any chondroitin sulfate, but hyaluronate and heparitin sulfate.

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‡ We thank Dr. G. C. Godman for permission to use these data.

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# Establishment of Resistance to Growth of Ehrlich Ascites Carcinoma in C57 Black Mice.* (25328)

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The Ehrlich carcinoma, growing either as an ascites or solid cancer in mice, is known to kill nearly all its hosts. Our observation of the survival of about one C57BL mouse out of each 100 animals, following an initial and repeated injections of large doses of viable carcinoma cells, led us to consider the possibility of producing the resistance at will. We have been able to produce in C57BL mice a resistance to viable Ehrlich ascites carcinoma cells by repeated injection of x-irradiated Ehrlich cells. Likewise, Donaldson and Mitchell (1) have recently reported protection of mice against growth of Ehrlich ascites tumor employing x-irradiated cells. Revesz(2), working with a number of different cancer cells, including Ehrlich's carcinoma, showed that implantation of an inoculum of viable cells admixed with a larger amount of the cancer cells lethally damaged with x-rays, caused an increase in cancer growth in highly inbred mice strains, while there was a slowing of cell growth when injected into genetically incompatible animals.

*Materials and methods.* The mice used in this study were C57BL males and females produced in our vivarium by indiscriminate interbreeding of stock obtained from the Roscoe B. Jackson Memorial Labs., Bar Harbor, Maine, about 4 years ago. At start of experiments the mice were 8-16 weeks of age. The Ehrlich ascites carcinoma was obtained 7 years ago from the Massachusetts Gen. Hosp., Boston, and since then has been grown in our laboratory by weekly 0.1 ml intraperitoneal and less frequent subcutaneous passages through C57BL mice. Ascites carcinoma grown intraperitoneally for 7 days was employed for the studies. The liquid tumor was withdrawn from the peritoneal cavity under sterile conditions and employed untreated for injection or placed in special sterile flasks for rotation under the x-ray machine. Two to 3 ml of ascites tumor were placed in these 50 ml balloon flasks and rotated at a rate of 30 rpm. The tumor was irradiated with 2000, 4000 and 8000 r, at a rate of about 50 r per minute and a focal distance of 45 cm. Dose rate was calibrated in air for the 250 kv x-ray head having 0.21 mm of Cu, inherent. Additional filters were 1 mm of aluminum and 0.5 mm of Cu, parabolic. The half value layer was 2.1 mm of Cu. All tumor injections into the mice were made intraperitoneally with Ehrlich ascites carcinoma containing about 100,000 cancer cells per mm$^3$. All challenges were made with 0.05 ml of ascites carcinoma. Metabolic studies were carried out on the carcinoma cells following a 3-fold washing with cold isotonic 55 mM phosphate solution (3). Oxygen consumption measurements were performed as previously described(3) employing glucose as the substrate at 38°C. Glucose utilization determinations were made at intervals of 30 minutes on tumor cells incubated at 38°C employing the methods of Somogyi(4) and of Nelson(5).

*Results.* The data in Table I indicate that little resistance to growth of the cells in mice is produced until 5 or more injections of x-irradiated tumor were given. There appear to be only small differences in the results obtained employing 5 to 10 injections of x-irradiated tumor. As indicated in Table II, varying amounts of the 2000 r irradiated tumor (0.025 to 0.5 ml) were tested. There were no discernible differences in resistance produced when employing 8 injections of .025, .05 or .1 ml of x-irradiated tumor. One injection of .05 ml produced no effect, however at .2 and .5 ml there appeared to be some protection (Table II). The .5 ml dose is about the maximum amount tolerated, since the mice developed large quantities of tumor with grossly distended abdomens. However, the

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TABLE I. Production of Resistance in C57 Black Mice to Ehrlich Ascites Carcinoma Cells. (0.05 ml Ehrlich ascites carcinoma cells x-irradiated with 2000 r and injected intraperitoneally once each week.)*

| | | Animals surviving challenges† | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | (No. of challenges, non-x-irradiated cells) | | | | | | | | | |
| No. of mice | No. of inj. x-irrad. cells | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| | | (No. of animals) | | | | | | | | | |
| 6 | 1 | 0 | | | | | | | | | |
| 4 | 2 | 0 | | | | | | | | | |
| 4 | 3 | 3 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | | |
| 2 | 4 | 0 | | | | | | | | | |
| 4 | 5 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | | |
| 4 | 6 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | | |
| 42 | 8 | 40 | 26(2)‡ | 20 | 19 | 19 | 19(4)‡ | 15 | 15(1)‡ | | |
| 20 | 10 | 20 | 19 | 17 | 16 | 16 | 16 | 15(1)‡ | 14 | 14 | 13(1)‡ |

* Control groups are not indicated but each week 15-25 mice were inj. with 0.05 to 0.20 ml of Ehrlich ascites carcinoma and 99% of the animals died with avg time of death about 14 days.

† All challenges were made with 0.05 ml of ascites carcinoma containing about 100,000 cancer cells/mm$^3$.

‡ No. in parentheses are No. of animals sacrificed for complement fixation studies.

animals were able to control their tumor growth. Table III indicates that between 2000 and 4000 r is the optimum amount of x-irradiation under the conditions employed. If less than 2000 r were employed there was too little alteration of the cells and the mice died of cancer. Various procedures were employed in unsuccessful attempts to produce altered cells which would be effective in producing resistance in mice. These attempts included freezing and thawing, grinding with a Ten-Broeck tissue grinder, ultra-sonic vibration and lyophilizing. Either there was inadequate alteration as in the first 3 conditions and the cancer cells overwhelmed the mice or the cells were completely destroyed and the mice showed no resistance, as was observed with lyophylizing.

TABLE II. Influence of Size and Number of Injections on Production of Resistance in C57 Black Mice to Ehrlich Ascites Carcinoma Cells. (Ehrlich ascites carcinoma cells were x-irradiated with 2000 r and inj. intraper. twice each week.)

| | | | Animals surviving challenges | | | |
|---|---|---|---|---|---|---|
| | | | (No. of challenges, viable cells) | | | |
| No. of mice | No. of inj. x-irrad. cells | Size of dose (ml) | 0 | 1 | 2 | 3 |
| | | | (No. of mice) | | | |
| 5 | 8 | .025 | 5 | 5 | 2 | 2 |
| 5 | 8 | .05 | 5 | 5 | 2 | 2 |
| 5 | 8 | .10 | 5 | 5 | 2 | 2 |
| 26 | 1 | .20 | 26<br>(22)* | 1 | 0 | |
| 6 | 1 | .50 | 6<br>(4)* | 1 | 0 | |

* Sacrificed to determine tumor volume.

Before first challenge of those receiving the 0.20 ml dose 3 mice died, and of those receiving the 0.50 ml dose 1 mouse died. These deaths were due to inadequate x-irradiation of the tumor.

TABLE III. Influence of Varying X-ray Dosage on Production of Resistance in C57 Black Mice to Ehrlich Ascites Carcinoma Cells. (0.05 ml of Ehrlich ascites carcinoma cells x-irradiated with 2000 r and inj. intraper. twice each week.)

| | | | Animals surviving challenges | |
|---|---|---|---|---|
| | | | (No. of challenges, viable cells) | |
| No. of mice | No. of inj. x-irrad. cells | Amt of x-irrad. (r) | 1 | 2 |
| 6 | 8 | 2000 | 6 | 4(1)* |
| 6 | 8 | 4000 | 6 | 2(2)* |
| 6 | 8 | 8000 | 0 | |

* Animals sacrificed for complement fixation studies.

Table IV shows the slow rate of growth and eventual control of the injected irradiated tumor, compared to the rapid rate of cell growth with non-treated cells. Average survival time of the latter was about 14 days. It was determined that the x-irradiated tumor growing in mice for 2 to 6 days and being controlled by the host would, when injected into non-resistant mice, kill within 18 to 23 days. This shows a remarkable recovery of viability of x-irradiated cells as well as a production of resistance in the first host.

Table V indicates some alteration of metabolism of the cancer cells produced with various amounts of x-irradiation. It is interesting that there was inhibition of oxygen con-

TABLE IV. Growth of X-Irradiated and Non-Irradiated Ehrlich Ascites Carcinoma Cells. (Single 0.2 ml intraper. inj. of ascites tumor, 0.05 ml cells.)

| Treatment of tumor, r | Vol of tumor cells (ml)<br>Days after inj.<br>2 | 4 | 5 | 6 | 7 | 8 | 9 |
|---|---|---|---|---|---|---|---|
| None | .20 | .50 | .66 | 1.06 | 1.15 | 1.25 | 1.90 |
| 2000 | .20 | .21* | .22* | .23* | .13 | .13 | .12 |

* Tumor from these 3 animals which had received the 2000 r tumor, when inj. into non-resistant mice killed in 19, 23 and 18 days, respectively. A second serial passage of the regressing tumor killed the mice in 14 to 16 days. Avg survival time of mice given non-treated and viable tumor survived an avg of 14 days.

sumption when the cells were treated with 500 r, and stimulation when greater amounts of irradiation were employed. Glucose consumption on the other hand was stimulated by irradiation particularly at 2000 r. Microscopic examination of the irradiated cells indicated the nuclei to be altered markedly. More detailed enzymatic studies are being carried out to evaluate more completely the cellular changes effected by x-irradiation.

Furthermore, preliminary studies show that 8 injections of x-irradiated Ehrlich ascites carcinoma cells also prevent growth of Ehrlich cancer cells injected subcutaneously in C57 black mice.

*Discussion.* Other technics have been applied with less success for production of immunity in the host animal carrying a tumor. Fink, Smith and Rothlauf(6) were able to show production of antibody in BALB/C mice following injection of lyophilized tumor S 621 in Freund's adjuvant. Hirsch, Bittner, Cole and Iversen(7) have reported that inbred strain C Bagg albino mice can be partially immunized against its own tumor, C 10040, employing the technic of Martinez(8). Also, Lund(9) has been able to vaccinate rats against Yoshida ascites sarcoma by injecting the viable carcinoma cells intracaecally; and Bradner, Clarke and Stock(10) stimulated the defenses of the female Swiss albino mouse to sarcoma 180 by injecting zymosan; Prince, Fardon, Nutini and Sperti(11) produced immunization with a transplantable mouse tumor. It is our belief that x-irradiation of the Ehrlich ascites carcinoma temporarily modifies the cells in such fashion as to alter and slow their growth and make them more antigenic. There is stimulation of formation of antibodies, which increases with repeated injections of the altered tumor. This is strongly indicated by the control of growth of the x-irradiated Ehrlich cells in the original mouse, and subsequent killing of the recipient mouse into which the regressing tumor is transferred. Further evidence for immunization of the C57BL mouse to the Ehrlich ascites carcinoma cell are our preliminary complement fixation analyses showing high titres of antibodies in the blood of the mice to the ascites carcinoma cells.

TABLE V. Influence of X-Irradiation on Oxygen Consumption and Glucose Utilization of Ehrlich Ascites Carcinoma Cells.

| Treatment of cells (r) | Glucose<br>(μM/0.04 ml cells/hr) | Oxygen |
|---|---|---|
| 0 | 4.1 | 3.1 |
| 500 | 4.3 | 2.6 |
| 1000 | | 3.0 |
| 2000 | 6.8 | 3.4 |

*Summary.* It has been possible to immunize C57BL mice against Ehrlich carcinoma by giving 5 to 10 injections of Ehrlich carcinoma x-irradiated with 2000 to 4000 r. Of 70 mice so immunized 66 survived the first challenge with viable cells; and all 35 of the mice receiving 8 challenges survived. It appears that the carcinoma cells are altered by x-irradiation and thus stimulate production of resistance to both x-irradiated and completely viable Ehrlich carcinoma cells.

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# Enhancement of Growth of Vit. $B_6$-Deficient Rats by Chlorpromazine Hydrochloride (SKF No. 2601-A) (25329)

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The beneficial effects of chloro-(3-dimethylamino-3′-propyl)-10-phenothiazine hydrochloride (chlorpromazine hydrochloride) on stressed, intact animals, as manifested by increased survival times under conditions of hemorrhagic shock(1,2,3,4,5), X-irradiation (6) and other types of stress(7) have been reported. The anti-stress results with this drug were not always consistent; however, differences in effects often appeared to be related to level of drug used. Chlorpromazine, at a level of 10 mg/kg/day, has been reported to retard growth of rats(8), and at a level of 3.4 mg/rat/day to increase urinary nitrogen excretion and also to reduce weight gain(9). The present study demonstrated that the drug may either enhance or depress growth, depending upon level administered and nutritional status of animal.

*Methods.* Weanling, Long-Evans strain rats were divided into 8 groups of 12 rats each (6 males and 6 females). Four groups were fed different levels of chlorpromazine in the absence of pyridoxine hydrochloride, and 4 groups served as controls at the same levels of drug in a complete diet (Table I). Composition of basal diet was as follows: casein (vit. free), 18%; sucrose, 65%; dextrose, 5%; salt mixture (USP XIV), 4%; alphacel, 4%; and cottonseed oil, 4%. Each kg of diet contained the following micronutrients (mg): thiamin hydrochloride, 10; riboflavin, 16; calcium pantothenate, 40; nicotinic acid, 100; 2-methyl-1,4-naphthoquinone, 1; biotin, 0.6; folic acid, 4; Vit. $B_{12}$, 0.05; and choline chloride, 1200. Each kg of diet also contained 10,000 USP units of Vit. A acetate, 1000 USP units of Vit. D and 125 mg of alpha-tocopherol acetate. All vitamins and chlorpromazine were added at the expense of sucrose except choline chloride, which was added at the expense of dextrose. Diets were fed *ad lib.* for 16 weeks. Animals were housed individually in metal cages with raised screen bottoms. Weight gains were recorded weekly and food consumption data were collected during second, sixth and fourteenth weeks. Studies on blood from tails of rats were made during fifth and sixth week of experiment. Standard analytical procedures were used on all blood tests (10). Studies were made on 24-hour urine samples collected from individual rats kept in metabolism cages during 2 periods of experiment: 1) 12-14 weeks and 2) 14-16 weeks after start of experiment. Urinary creatine and creatinine levels were measured by the method of Folin(11), while urinary phosphates were determined by the method of Fiske and Subbarow(12). Animals were sacrificed for autopsy by exsanguination while under light ether anesthesia at end of 16 weeks. Liver, kidneys, spleen, heart, adrenals, thymus, and testes were removed, trimmed, and weighed wet at time of autopsy. Livers were analyzed for Vit. $B_6$ by microbiological assay(13).

TABLE I. Additives to Basal Diet.

| Group No. | Pyridoxine hydrochloride, mg/kg diet | Chlorpromazine HCl (SKF No. 2601-A), mg/kg diet |
|---|---|---|
| I | None | 100 |
| II | ” | 200 |
| III | ” | 400 |
| IV | 10 | 100 |
| V | ” | 200 |
| VI | ” | 400 |
| VII | ” | None |
| VIII | None | ” |

*Results. Growth and food consumptions.* A significant improvement in growth of $B_6$-deficient rats occurred when chlorpromazine was included at 100 mg/kg of diet (Fig. 1, 2). This growth-promoting effect of the drug was less evident at the 200 mg/kg level and was absent at the 400 mg/kg level.

Weight gains of both male and female $B_6$-supplemented rats were less at all 3 levels of dietary chlorpromazine tested than in control rats (Group VII) (Fig. 3, 4). Food consump-

TABLE II. Average Amount of Food (g/Rat/Day) and SKF No. 2601-A (mg/kg Rat/Day) Consumed by Rats Receiving 100 mg Drug/kg Diet during 2nd, 6th and 14th Weeks.*

| | Food consumption (g/rat/day)† and mg SKF No. 2601-A consumed/kg rat/day‡ | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | B₆ deficient | | | | B₆ supplemented | | | |
| | Controls | | 100 mg SKF No. 2601-A/kg diet | | Controls | | 100 mg SKF No. 2601-A/kg diet | |
| Week | ♂ | ♀ | ♂ | ♀ | ♂ | ♀ | ♂ | ♀ |
| 2 | 7.2 ± .6 | 7.2 ± .7 | 6.3 ± .8<br>(9.1) | 6.0 ± .5<br>(9.9) | 8.2 ± .7 | 10.2 ± .7 | 7.6 ± .9<br>(8.7) | 8.6 ± .3<br>(10.4) |
| 6 | 7.6 ±1.5 | 6.4 ± .8 | 6.7 ± .4<br>(6.8) | 5.9 ± .3<br>(7.5) | 12.4 ±1.0 | 12.1 ± .5 | 12.8 ±1.0<br>(6.6) | 12.0 ± .4<br>(7.6) |
| 14 | 6.2 ± .4 | 7.0 ±1.0 | 7.6 ± .7<br>(5.1) | 6.8 ± .8<br>(5.9) | 15.2 ± .8 | 11.7 ± .7 | 15.7 ±1.1<br>(5.6) | 12.8 ±1.3<br>(7.0) |

* Samples from 16 wk feeding period. † Includes stand. error of mean. ‡ Values given in parentheses.

tions and amounts of drug consumed by male and female rats receiving the most active level of drug (100 mg chlorpromazine/kg of diet) during second, sixth and fourteenth weeks of experiment are presented in Table II. No significant differences in food consumption were observed between drug-supplemented and control groups at any periods used for food consumption measurements.

*Blood studies.* Chlorpromazine at the 3 die-

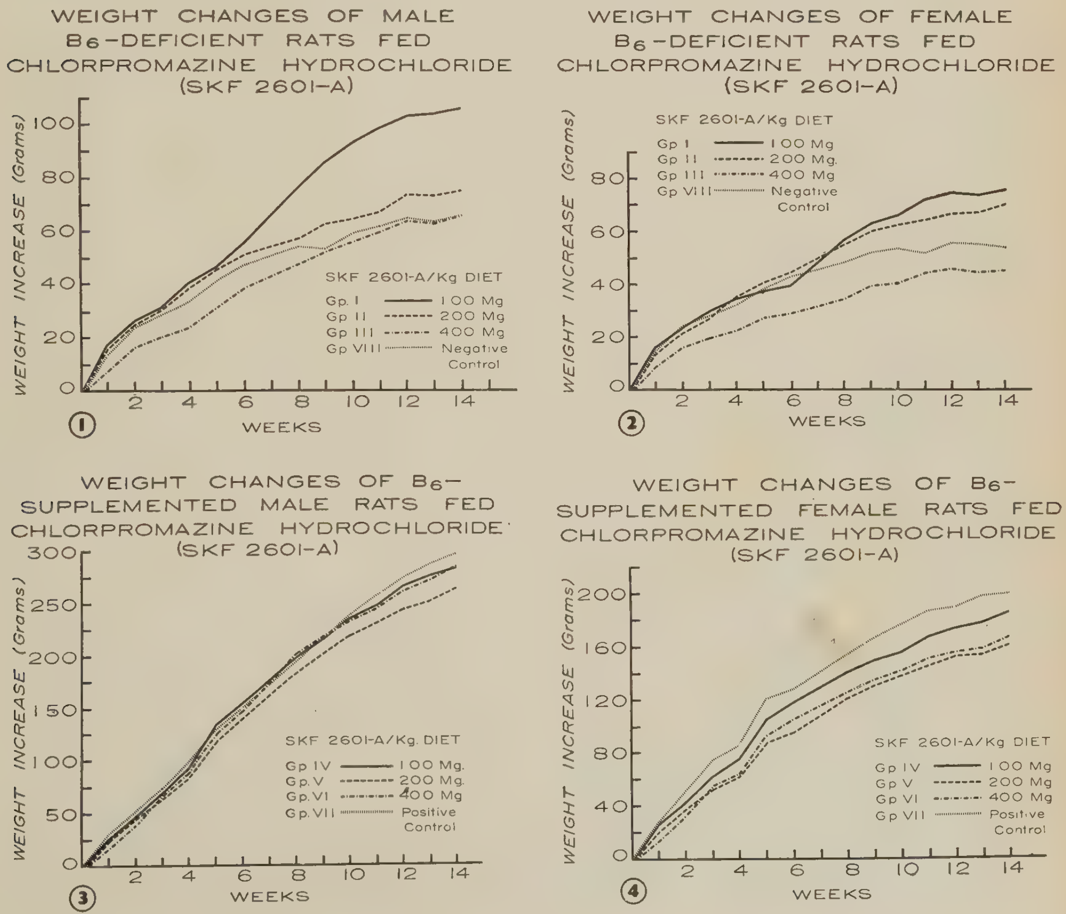

FIG. 1-4.

TABLE III. Summary of Blood Studies on $B_6$-Deficient and Control ($+B_6$) Rats With and Without Chlorpromazine HCl (SKF No. 2601-A).

| | Supplements | | | | | | Differential W.B.C.† | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Group No. | $B_6$ | SKF #2601-A, mg/kg diet | Hematocrit,* ml/100 ml | R.B.C.* × $10^6$/mm³ | Hemoglobin,* g/100 ml | Total† W.B.C./mm³ | Lymphocytes | Neutrophils | Eosinophils | Basophils | Monocytes |
| | | | | | | | % | % | % | % | % |
| | | | | | Males | | | | | | |
| I | — | 100 | 41 | 6.73 | 13.4 | 11,250 | 52 | 43 | 1 | 2 | 2 |
| II | — | 200 | 42 | 7.26 | 14.1 | 10,130 | 67 | 30 | .3 | 2 | .3 |
| III | — | 400 | 46 | 7.28 | 14.9 | 10,330 | 50 | 46 | 2 | 0 | 2 |
| IV | + | 100 | 39 | 5.97 | 13.5 | 10,780 | 65 | 32 | 1 | 0 | 1 |
| V | + | 200 | 38 | 6.39 | 13.4 | 10,930 | 72 | 25 | 1 | .3 | 1 |
| VI | + | 400 | 34 | 5.77 | 13.0 | 10,970 | 62 | 34 | 4 | 0 | .3 |
| VII | + | | 35 | 5.63 | 12.7 | 12,730 | 62 | 34 | 2 | 0 | 2 |
| VIII | — | | 38 | 5.60 | 13.2 | 13,330 | 51 | 47 | 0 | 0 | 2 |
| | | | | | Females | | | | | | |
| I | — | 100 | 42 | 8.48 | 14.2 | 13,800 | 67 | 26 | 3 | 0 | 5 |
| II | — | 200 | 41 | 8.04 | 13.8 | 11,400 | 54 | 37 | 3 | 0 | 7 |
| III | — | 400 | 45 | 8.07 | 14.3 | 10,600 | 70 | 25 | 3 | 0 | 3 |
| IV | + | 100 | 39 | 6.90 | 12.9 | 13,700 | 70 | 25 | 1 | 0 | 4 |
| V | + | 200 | 40 | 6.51 | 13.7 | 11,500 | 69 | 25 | 3 | 0 | 3 |
| VI | + | 400 | 41 | 6.38 | 13.6 | 13,200 | 77 | 16 | 3 | 0 | 4 |
| VII | + | | 42 | 6.68 | 14.5 | 10,600 | 77 | 14 | 3 | 0 | 6 |
| VIII | — | | 44 | 6.99 | 13.4 | 7,300 | 68 | 27 | 1 | 0 | 3 |

* Avg of 6 rats/group.

† Avg of 3 rats/group.

TABLE IV. Urinary Creatine/Creatinine Ratio for Vit. $B_6$-Deficient or $B_6$-Supplemented Rats Administered Chlorpromazine HCl (SKF No. 2601-A).

| Group No. | Vit. $B_6$ | SKF #2601-A, mg/kg diet | Creatine/Creatinine ratio* ♂ 12-14 wk | ♂ 14-16 wk | ♀ 12-14 wk | ♀ 14-16 wk |
|---|---|---|---|---|---|---|
| I | — | 100 | .38 ± .1 | .48 ± .2 | .97 ± .3 | 1.14 ± .5 |
| II | — | 200 | 1.29 ±1.0 | .52 ± .2 | 4.67 ±1.5 | 5.48 ±2.5 |
| III | — | 400 | 1.49 ±1.2 | 1.73 ±1.0 | 8.79 ±8.3 | 4.04 ±2.5 |
| IV | + | 100 | .10 ± .03 | .22 ± .08 | .26 ± .05 | .43 ± .2 |
| V | + | 200 | .12 ± .05 | .14 ± .06 | .33 ± .09 | .26 ± .06 |
| VI | + | 400 | .14 ± .04 | .10 ± .05 | .36 ± .03 | .25 ± .05 |
| VII | + | | .14 ± .04 | .06 ± .03 | .24 ± .03 | .25 ± .03 |
| VIII | — | | 1.00 ± .3 | .89 ± .3 | 3.93 ±1.7 | 2.24 ± .8 |

* Includes stand. error of mean.

tary levels (100, 200, and 400 mg/kg) increased red blood cell counts and hemoglobin values in male and female $B_6$-deficient rats, but did not affect normal rats (Table III). White blood cell counts were elevated in female $B_6$-deficient animals but were reduced in male $B_6$-deficient rats receiving chlorpromazine. This same trend toward alteration of WBC count was noticed in $B_6$-supplemented male and female rats receiving the drug. Calculations of mean corpuscular volume and mean corpuscular hemoglobin revealed that red cells of $B_6$-deficient rats receiving chlorpromazine were consistently smaller and contained less hemoglobin than did $B_6$-deficient controls.

*Urine studies.* Creatinine excretion was related to food intake in both $B_6$-deficient and normal ($B_6$-supplemented) rats. Creatine excretion was elevated above normal levels in control $B_6$-deficient male and female rats (Group VIII); however, the lower level of chlorpromazine (Group I) induced a significant reduction of creatine urinary excretion. In $B_6$-supplemented rats, receiving chlorpromazine, creatine levels were not different from those of normal control rats (Group VII). These results are reflected in the creatine/creatinine ratios reported in Table IV. A tendency for all 3 levels of chlorpromazine to lower phosphate excretion in both $B_6$-deficient and control male rats was also noted; however, this trend was not observed for female rats.

*Autopsy data.* Hearts and adrenal glands of male and female $B_6$-deficient rats were more nearly normal in weight (smaller) in animals receiving chlorpromazine (Groups I, II and III) than they were in negative controls (Group VIII, Table V). Gonads of both male and female $B_6$-deficient rats receiving chlorpromazine (especially on lower level of drug [Group I]), and uteri of female rats were increased in weight over those of $B_6$-deficient controls.

*Liver $B_6$ levels.* Chlorpromazine did not significantly alter storage level of Vit. $B_6$ in livers of male or female $B_6$-deficient rats or in normal rats.

*Discussion.* In $B_6$-deficient rats receiving low levels of chlorpromazine, an increase in body weight occurs with only a slight increase in food consumption. The more normal relative organ weights of rats on pyridoxine-deficient diets containing low levels of chlorpromazine indicate an increase in available Vit. $B_6$ for utilization by the animals. In deficient animals the drug also reversed to a large extent the loss of metabolically important urinary creatine and phosphates. These activities are in all respects similar to those of adding vitamin to the diet of animals.

Similar levels of drug in Vit. $B_6$-supplemented rats had no effect in our tests other than to cause some depression in weight gain. Whether much lower doses of chlorpromazine would increase the weight of Vit. $B_6$-supplemented rats is not known. Observations have been made in our Microbiology Section (unpublished) that very low levels of chlorpromazine stimulate growth of *L. acidophilus,* whereas higher levels of drug inhibited growth.

Although there was no increase of pyridoxine in livers of animals which showed enhancement of body weight when fed a pyridoxine-deficient diet with chlorpromazine HCl, utili-

TABLE V. Autopsy Data on Rats Sacrificed at End of Experiment.

| | Supplement | | Avg relative organ weights (per 100 g of body wt) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Males | | | | | | | |
| Group No. | SKF 2601-A, mg/kg diet | Vit. $B_6$ | Liver, g | Kidneys, g | Spleen, mg | Heart, mg | Adrenals, mg | Thymus, mg | Testes, g | |
| I | 100 | — | 3.81 | .85 | 190 | 378 | 15.7 | 32.6 | 1.43 | |
| II | 200 | — | 3.66 | .83 | 160 | 336 | 14.7 | 26.1 | 1.34 | |
| III | 400 | — | 4.32 | 1.01 | 200 | 378 | 20.5 | 34.7 | 1.30 | |
| IV | 100 | + | 3.48 | .59 | 150 | 275 | 8.6 | 74.9 | .89 | |
| V | 200 | + | 3.70 | .76 | 180 | 299 | 10.4 | 72.6 | .99 | |
| VI | 400 | + | 3.38 | .58 | 150 | 277 | 9.8 | 64.9 | .81 | |
| VII | | + | 3.44 | .62 | 160 | 250 | 11.1 | 61.9 | .93 | |
| VIII | | — | 4.01 | 1.18 | 240 | 435 | 25.2 | 47.5 | 1.17 | |
| | | | Females | | | | | | | |
| | | | Liver, g | Kidneys, g | Spleen, mg | Heart, mg | Adrenals, mg | Thymus, mg | Ovaries, mg | Uterus, mg |
| I | 100 | — | 4.24 | .85 | 180 | 438 | 22.6 | 25.1 | 45.0 | 103.1 |
| II | 200 | — | 4.34 | .98 | 260 | 361 | 19.4 | 32.2 | 25.2 | 28.4 |
| III | 400 | — | 4.27 | 1.10 | 270 | 389 | 24.3 | 47.7 | 31.0 | 49.4 |
| IV | 100 | + | 3.29 | .64 | 180 | 296 | 19.4 | 82.2 | 39.4 | 114.6 |
| V | 200 | + | 3.69 | .72 | 270 | 386 | 15.8 | 84.0 | 42.3 | 144.3 |
| VI | 400 | + | 3.55 | .71 | 200 | 282 | 17.6 | 105.6 | 41.7 | 186.7 |
| VII | | + | 2.91 | .63 | 170 | 262 | 18.6 | 104.8 | 30.5 | 141.4 |
| VIII | | — | 4.47 | 1.24 | 270 | 551 | 27.5 | 44.4 | 32.7 | 43.4 |

zation of any increased level of vitamin by the animal might have obscured the fact that under the influence of the drug, more vitamin was being made available to the animal. The recent work of Barnes *et al.*(14) indicates that coprophagy may be the mechanism by which the animal obtains increased vitamins synthesized in the cecum or large intestine rather than by direct absorption. This factor is under investigation.

Work has been completed in our laboratory in which chlorpromazine HCl either inhibited or potentiated activity of yeast hexokinase depending on concentration of drug. These observations, to be reported, indicate that the drug may be acting to protect the animal at some points in its metabolic function from loss or wastage of nutrients and energy.

The fact that low levels of the drug are active as stimulants, to 'growth' similar to findings with a number of other stimulants(15), and that these effects are observed in a deficient diet but not in a complete diet make this phenomenon closely similar to that with antibiotic as a 'growth' enhancing agent(16).

*Summary.* At very low dietary levels, chlorpromazine has a favorable effect on growth and metabolism of Vit. $B_6$-deficient rats. These animals gained more weight, had more nearly normal organ weights, and excreted less creatine and phosphates than did the pyridoxine-deficient controls.

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## Plasma Unesterified Fatty Acid Concentration in Fetal and Neonatal Life.* (25330)

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During late stages of pregnancy the human fetus rapidly builds up fat stores in adipose tissue. High respiratory quotients during this period suggest catabolism of carbohydrate or synthesis of fat, or both(1). Decrease in respiratory quotient to approximately 0.7(2) and loss of weight usually seen in first few days of life suggest that these lipid stores are drawn on for energy needs in neonatal life. Utilization of lipid is not surprising since glycogen stores are not overly abundant in the newborn(2) and since protein catabolism can account for only a fraction of caloric requirement during first 48 hours of life(3). Recent studies on metabolic activity and physiological significance of plasma unesterified fatty acids (UFA) in adult mammals indicate that UFA originate chiefly in adipose tissue and appear to be the major form of transported lipid immediately available for oxidation by peripheral tissues(4). The plasma level of UFA is decreased under conditions that promote oxidation of carbohydrate, and increased when carbohydrate is less readily available for energy needs(4). These considerations led us to investigate metabolism of UFA in fetal and neonatal life. Our report describes plasma levels of UFA in sheep fetus and in newborn man and sheep during first few hours of life. Plasma UFA concentrations in mothers were also measured concomitantly.

*Methods. Studies in sheep.* In sheep the uterus can be opened and umbilical vessels cannulated without apparent disturbance of the placental function(5). Cesarean sections under chloralose anesthesia were performed on 4 crossbred sheep within a few days of term. The fetuses were delivered and immediately immersed without interruption of placental circulation in bath of Ringer's solution maintained at 39°C. Polyethylene catheters were placed in tributaries of the umbilical artery and vein and threaded into main channels. A catheter was also placed in carotid artery of the mother. Serial blood samples were obtained simultaneously from these 3 sources over 1 to 3 hr. period. In one case the fetus was delivered successfully from the incubating bath and serial blood samples were obtained from the umbilical artery for 6 hours. *Studies in man.* Mothers of infants received a variety of analgesic and anesthetic agents during labor. Single blood samples were obtained from the umbilical vein and artery of 9 newborn infants and from an antecubital vein of their mothers. All samples were taken within 2 min. after delivery and before separation of the placenta (Group 1). In an additional 8 cases, samples were taken from either the umbilical vein or artery of the infant and from the antecubital vein of the mother 1 to 7 min. after delivery (Group 2). Single blood samples were also obtained from the internal jugular vein of 9 newborn infants 1 to 6½ hr after birth (Group 3).§ These samples were

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† Postdoctoral Research Trainee in Obstetrics and Gynecology.

‡ Established Investigator of Am. Heart Assn.

§ We are indebted to Dr. William Tooley for obtaining these samples. In adults we found (unpublished) that UFA concentrations of internal jugular and arterial blood plasma are comparable.

TABLE I. Unesterified Fatty Acid Concentration (UFA) in Fetal and Maternal Blood Plasma of Sheep. (Expressed as mean and range.)

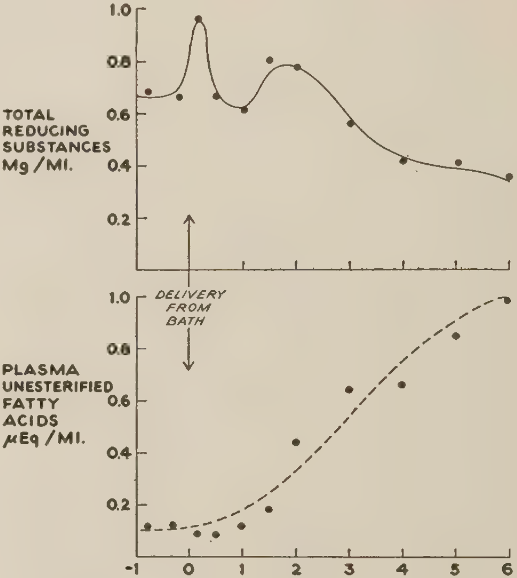
| | Exp. 1 | | Exp. 2 | | Exp. 3 | | Exp. 4 | |
|---|---|---|---|---|---|---|---|---|
| Source | No. samples | UFA, $\mu$eq/ml | No. samples | UFA, $\mu$eq/ml | No. samples | UFA, $\mu$eq/ml | No. samples | UFA, $\mu$eq/ml |
| Umbilical vein | 8 | .14<br>(.10- .18) | 7 | .12<br>(.10-.15) | 4 | .12<br>(.08-.20) | 4* | .13 |
| Umbilical artery | 8 | .15<br>(.10- .23) | 9 | .12<br>(.08-.18) | 4 | .15<br>(.08-.26) | 4* | .14 |
| Maternal artery | 10 | 1.14<br>(.98-1.32) | 15 | .42<br>(.30-.63) | 6 | .38<br>(.26-.52) | 9 | 1.0<br>(.82-1.12) |

* Pooled samples.

obtained within 5 min. of taking the infant from the crib. In an additional 2 infants single blood samples were obtained from femoral vein 6 hr after delivery. The infants received no nutrients prior to blood sampling. *Analytical.* All blood samples were mixed with 1.5 mg/ml of sodium oxalate, chilled in ice and centrifuged at 4°C. Plasma was analyzed immediately or stored at –19°C. UFA were extracted from plasma by the method of Davis(6). Ether extracts were evaporated under air stream at room temperature, taken up in heptane, and titrated according to the method of Dole(7). Total reducing substances were determined by the method of Somogyi (8) and glucose by the method of Huggett and Nixon(9).

*Results. In sheep (Table I).* In the fetuses plasma levels of UFA were uniformly low. Since titration values in extracts of fetal plasma frequently did not exceed twice the blank titration value, these results must be considered approximations of the true concentrations. No detectable difference was found between samples obtained from umbilical artrey and vein. Maternal plasma levels of UFA were much higher than fetal levels in all cases, but showed considerable individual variation. In one newborn lamb (Exp. 4, Table I) UFA concentration showed a striking rise, beginning 1 hr after delivery and continuing for 6 hr. Then it had increased tenfold (Fig. 1). During this period the concentration of total reducing substances fell considerably.

*In man.* As in fetal sheep, plasma levels of UFA were considerably lower in newborn infants in Group 1 than in their mothers (Table II). Comparable levels were obtained in Group 2. In mothers of the latter group the



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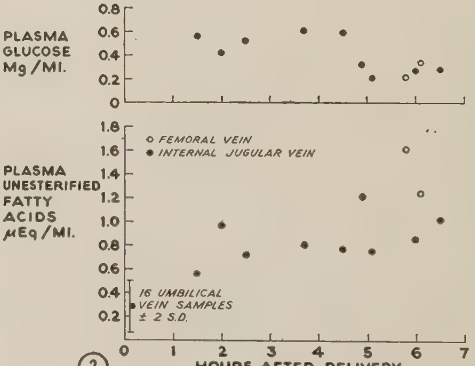

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FIG. 1. Changes in the concentration of total reducing substances and UFA in blood plasma of a lamb prior to and after delivery. Nikethamide, 125 mg, was inj. into the umbilical artery at time of delivery.

FIG. 2. Changes in the concentration of glucose and UFA in blood plasma of newborn infants.

TABLE II. Unesterified Fatty Acid Concentrations in Plasma of Newborn Infants and Their Mothers.

| Sec. after delivery | Unesterified fatty acids, $\mu$eq/ml | | | Medications and mode of delivery |
|---|---|---|---|---|
| | Umbilical vein | Umbilical artery | Maternal vein | |
| 10 | .20 | | | 100 mg pentobarbital<br>Cesarean section<br>Spinal anesthesia |
| 60 | | .20 | .96 | |
| 30 | .26 | | | Diabetic<br>100 mg pentobarbital<br>10 mg morphine<br>.4 mg scopolamine<br>Cesarean section<br>Spinal anesthesia |
| 120 | | .17 | .98 | |
| 30 | .25 | | .80 | 75 mg meperidine<br>Vaginal delivery<br>Local anesthesia |
| 120 | | .10 | | |
| 30 | .16 | | | No medication<br>Vaginal delivery<br>Local anesthesia |
| 150 | | .22 | .88 | |
| 40 | .24 | | | 200 mg secobarbital<br>Trichloroethylene<br>Vaginal delivery<br>Local anesthesia |
| 180 | | .33 | .85 | |
| 120 | .37 | | | 50 mg meperidine<br>25 mg promethazene<br>Vaginal delivery<br>Local anesthesia |
| 180 | | .26 | .90 | |
| 120 | .50 | | | *Idem* |
| 180 | | .25 | .90 | |
| 120 | .20 | | | No medication<br>Vaginal delivery<br>Local anesthesia |
| 240 | | .15 | .85 | |
| 180 | .26 | | | 100 mg meperidine<br>Vaginal delivery<br>No anesthesia |
| 300 | | .26 | .98 | |
| Mean | .27 | .22 | .90 | |
| S.D. | .11 | .07 | .06 | |

highest concentration of UFA (1.38 meq/l) was found in a patient who had completed a prolonged labor. UFA concentration of plasma from the umbilical vein of the infant of this mother, however, was not significantly higher than that of other newborns. UFA concentrations were usually lower in samples obtained from the umbilical artery than from the umbilical vein, but this difference was not statistically significant ($.4 > p > .3$).

Plasma UFA concentrations showed no significant change during the first 7 min. of extra-uterine life. The levels had risen significantly, however, 2 hr after birth and continued to increase up to 6½ hr. Plasma glucose concentration was low when first measured 1½ hr after birth and was strikingly reduced after 5 to 7 hr (Fig. 2).¶

*Discussion.* Low plasma levels of UFA in fetal sheep and newborn infants are consistent with the concept that the mammalian fetus obtains the bulk of its energy requirements from catabolism of carbohydrate. The levels in fetal sheep are the lowest noted consistently in any situation yet reported(4). Existence of a large difference in concentration of plasma UFA in the fetal and maternal circulations suggests that the placenta might not be freely permeable to UFA. However, in preliminary studies (unpublished) palmitic acid-1-$C^{14}$ was shown to cross placental membranes in sheep and rabbits rapidly.

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¶ Published data indicate that plasma glucose concentration is within normal limits at birth(2).

The increase in plasma UFA concentration during the first few hours after birth strongly suggests that UFA are rapidly mobilized from adipose tissue stores and serve to supply the energy needs of the organism. The associated hypoglycemia points to deficient carbohydrate oxidation as the probable major factor responsible for increased UFA concentration. An increase in sympathetic nervous activity could also account in part for the increase observed during the first few hours of life(10). The influence of various medications administered to mothers during labor cannot be defined fully, but it should be noted that the changes in UFA concentration were consistent in all infants studied.

The results presented raise the question of the effects of possible defective mobilization of UFA on survival of the full-term infant. The premature infant, furthermore, may lack sufficient stores of fat in adipose tissue to supply adequate quantities of UFA to vital organs. Further studies will be required to answer these and other questions raised by this investigation, such as source and fate of plasma UFA in the fetus, factors controlling the plasma level of UFA in the fetus and newborn, and the contribution of UFA to energy metabolism in fetal and neonatal periods.

*Summary.* Concentration of UFA in blood plasma of fetal sheep and newborn man was consistently much lower than in plasma obtained simultaneously from their mothers. The levels were comparable to those found in man after administration of glucose or insulin. In both species the plasma level of UFA rose rapidly within 2 hr of birth with an associated hypoglycemia. These findings support previous studies which suggested the occurrence of a rapid shift from carbohydrate to fat catabolism in the neonatal period.

We are greatly indebted to Drs. Louis Holm and Harold Parker of the University of California School of Veterinary Medicine, Davis, California, for their advice and assistance in the sheep studies.

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# Isolation and Characterization of Glucuronic Acid Conjugates of Chlorpromazine in Human Urine. (25331)

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Salzman and Brodie(1) and independent investigation by this laboratory(2) showed chlorpromazine sulfoxide to be a metabolite of chlorpromazine in the dog and man. Ross, Young and Maass(3,4) reported demethylation of chlorpromazine in the rat, indicating the existence of demethylated products of chlorpromazine and possibly chlorpromazine sulfoxide as metabolic products of chlorpromazine. Walkenstein and Seifter(5) reported the presence of mono-demethylated products of promazine and promazine sulfoxide in urine of dogs treated with promazine. Hydrolysis of dog and human urine by acid, alkali or $\beta$-glucuronidase treatment increases the amount of phenothiazine-like extractable material(2, 6). The conjugated material represents a large fraction of chlorpromazine metabolites excreted in the urine. In this study, we have isolated such material from the urine of pa-

TABLE I. Paper Chromatographic Pattern of Urinary Chlorpromazine Metabolites.

| Group | Phenothiazine metabolites | Endogenous constituents found also in control urine | Color developed by $HNO_2$ spray | Avg Rf |
|---|---|---|---|---|
| A. Rf <0.4 | $P_1$ | | blue | .01 |
| | $P_2$ | | purple | .03 |
| | $P_3$ | | " | .05 |
| | | $E_1$* | blue | .23 |
| | $P_4$ | | pink | .33 |
| B. Rf >0.4 | $P_5$ | | pink | .44 |
| | $P_6$ | | blue | .53 |
| | P mixture† | | purple-pink | .57–.85 |
| | | $E_2$‡ | pink | .95 |

* Constantly present. † Appears to be overlapping of at least 3 spots. ‡ Observed occasionally.

tients on chlorpromazine therapy and have characterized it as glucuronic acid conjugated chlorpromazine metabolites.

*Materials and methods. Chlorpromazine urine.* Pooled urine samples from hospital patients receiving 200-800 mg of chlorpromazine hydrochloride daily were preserved with toluene and refrigerated. Urine from normal individuals who had not received any phenothiazine served as control. *Paper chromatography of whole urine.* A descending paper chromatogram of 100 μl of urine was developed overnight on Whatman 3 MM paper with an "isoamyl" solvent system consisting of isoamyl alcohol, water, ethyl alcohol and formic acid in a volume ratio of 100:100:15:10. The paper was sprayed with a nitrous acid reagent consisting of concentrated hydrochloric acid containing a trace of $NaNO_2$. (All of a large number of phenothiazine compounds tested with this reagent developed colors—orange, pink, purple, violet and blue.) The development of colored spots thus indicated phenothiazine metabolites in the urine sample. *Separation of chlorpromazine metabolites by cation exchange resin. Preparation of column.* A slurry of Dowex 50 (200-400 mesh) in hydrogen form was poured into a 2 x 22 cm column to a height of 16 cm, and 100 ml of urine was applied followed by 100 ml of water. Gradient elution was carried out by coupling a flask containing 1000 ml of 1.4% ammonium hydroxide to another flask containing 1000 ml of water. *Examination of fractions.* A few drops from each collection tube were tested with a fresh $HNO_2$ reagent (12 ml of concentrated HCl + 1 ml of 0.01% $NaNO_2$ solution) for possible phenothiazine metabolites. Eluates that produced the same response to the $HNO_2$ reagent and appeared to contain the same phenothiazine(s) were pooled. Each of the pooled fractions was reduced *in vacuo* to a volume of approximately 25 ml, and a 100 μl aliquot of the concentrate was chromatogrammed with the "isoamyl" system described for whole urine. *β-glucuronidase hydrolysis.* A 5.0 ml aliquot was adjusted to pH 4.5 and incubated with 1.0 ml (5000 units) of mammalian β-glucuronidase at 37°C for 24 hours. A 100 μl aliquot was chromatogrammed. The pooled fractions, before and after hydrolysis, were analyzed for free and conjugated glucuronic acid by the method of Fishman(7). *Demonstration of released glucuronic acid.* The hydrolysates from the above fractions were treated with ion exchange resins to remove interfering material and chromatogrammed on 3 MM paper with a butyl alcohol, acetic acid and water (5:4:4) system. The paper was sprayed with an alkaline silver nitrate reagent and compared to the paper chromatograms of pure glucuronic acid and glucuronolactone.

*Results.* Paper chromatographic pattern of urinary chlorpromazine metabolites is shown in Table I. Group A phenothiazine metabolites ($P_{1\text{-}4}$) were not readily extractable from an aqueous medium by non-polar organic solvents.

The results of the separation of Group A from Group B chlorpromazine metabolites by Dowex 50 are shown in Table II. Group A metabolites were eluted from the cation exchange resin. Phenothiazine metabolites $P_1$,

TABLE II. Separation of Urinary Chlorpromazine Metabolites by Dowex 50.

| Fractions from Dowex 50 column | $HNO_2$ test | Rf |
|---|---|---|
| Urine effluent | Negative (only non-phenothiazine urinary constituents) | |
| Water wash | Blue, endogenous constituent $E_1$ | .23 |
| Tube No. 2-5 | Pink, " " $E_2$ | .95 |
| " " 35-50, *Fraction $P_{1\text{-}3}$* | Blue, metabolite $P_1$ | .03* |
| | Purple, " $P_2$ | .05* |
| | " " $P_3$ | .10* |
| " " 51-79 | Negative | |
| " " 80-110, *Fraction $P_4$* | Pink, metabolite $P_4$ | .33 |
| " " 111-200 | Negative | |

* These Rf's are higher than those found in whole urine (Table I), probably as a result of elimination of urinary constituents.

$P_2$ and $P_3$ were eluted as a mixture, Fraction $P_{1\text{-}3}$; $P_4$ was eluted separately, Group B metabolites were retained by the resin.

Both Fractions $P_{1\text{-}3}$ and $P_4$ contained conjugated glucuronic acid but no free glucuronic acid or glucuronolactone. When similar fractions of control urine collected from Dowex 50 were tested, only the fraction corresponding to Fraction $P_{1\text{-}3}$ contained conjugated glucuronic acid. Conceivably, Fraction $P_{1\text{-}3}$ contained both phenothiazine glucuronides and other urinary glucuronides. Fraction $P_4$ contained only phenothiazine glucuronides.

$\beta$-glucuronidase hydrolysis of Fractions $P_{1\text{-}3}$ and $P_4$ resulted in disappearance or reduction in quantity of metabolites $P_{1\text{-}4}$. Three new phenothiazines were observed on chromatographing Fraction $P_{1\text{-}3}$ hydrolysate and 2 in Fraction $P_4$ hydrolysate. These compounds were different from chlorpromazine and chlorpromazine sulfoxide. The effects of $\beta$-glucuronidase hydrolysis are recorded in Table III. Some of these released phenothiazines were extractable by ether at pH 13. The extract from Fraction $P_{1\text{-}3}$ hydrolysate showed a UV spectrum very similar to that of a mixture of chlorpromazine and chlorpromazine sulfoxide. The extract from Fraction $P_4$ showed a spectrum similar to that of chlorpromazine, with addition of a secondary absorption peak at 284 m$\mu$. This is suggestive of a ring hydroxyl function. In contrast to chlorpromazine and chlorpromazine sulfoxide, these phenothiazines were also extractable from a weakly acidic medium at pH 5.

TABLE III. Effect of $\beta$-Glucuronidase Hydrolysis on Fractions $P_{1\text{-}3}$ and $P_4$ (Paper Chromatographic Patterns).

| | Before hydrolysis | | After hydrolysis | |
|---|---|---|---|---|
| Fraction | Color with $HNO_2$ | Rf | Color with $HNO_2$ | Rf |
| $P_{1\text{-}3}$ $P_1$ | Blue | .03 | Absent | |
| $P_2$ | Purple | .05 | Slightly affected | |
| $P_3$ | " | .10 | Absent | |
| | | | New phenothiazine spots: | |
| | | | Purple | .47 |
| | | | " | .75 |
| | | | Blue* | .97 |
| $P_4$ | Pink | .33 | Absent | |
| | | | New phenothiazine spots: | |
| | | | Purple | .57 |
| | | | " | .85 |

* Trace, occasionally observed.

Released glucuronic acid was found in the hydrolysates of both Fractions $P_{1\text{-}3}$ and $P_4$.

*Discussion.* Incubation under mild conditions with $\beta$-glucuronidase caused complete disappearance of 3 of the highly water-soluble metabolites and partial disappearance of the fourth. This observation accompanied by release of glucuronic acid and less polar, ether extractable phenothiazines indicated that these 4 metabolites excreted in urine were glucuronides of chlorpromazine. The extractability of the released phenothiazines from an acidic medium further indicated the presence of enolic or phenolic functions. These observations support the prevalent mode of detoxificaton of aromatic compounds through ring hydroxylation. The authors feel that the glucuronides reported here are conjugated products of glucuronic acid and phenolic metabolites resulting from hydroxylation of the phenothiazine nucleus and that hydroxylation constitutes an important aspect of chlorpromazine metabolism. Other metabolic changes in the phenothiazine nucleus and dimethyl propylamine side chain are inferred by the presence of 4 glucuronide spots. The differences among these metabolites may be the result of hydroxylation at different positions of the nucleus accompanied by oxidation to sulfoxides and demethylation of the side chain.

*Summary.* Four polar, water-soluble metabolites of chlorpromazine were separated from urine by use of a cation exchange resin. These were shown to be glucuronides by release of free glucuronic acid and less polar, ether-extractable phenothiazines as a result of β-glucuronidase hydrolysis. Presence of enolic functions in these released phenothiazines was shown by their extractability from an acidic medium. Results strongly suggest hydroxylation of chlorpromazine and subsequent conjugation with glucuronic acid as an important route of chlorpromazine metabolism and detoxification in man.

Acknowledgement is made to Edward L. Kean for his earlier work that proved helpful, Dr. Karl Rickels for kind cooperation in providing urine specimens from his hospital ward, and Peter A. Simkin for technical assistance.

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## Metabolism of Propionate-1-C-14 in the Intact Mouse.* (25332)

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Previous *in vitro* studies indicated that adipose tissue may play an unique role in metabolism of propionic acid(1). It was noted, for instance, that incorporation of propionate-1-C-14 and propionate-2-C-14 into fatty acids of adipose tissue slices was 100 to 200 times the incorporation into fatty acids of liver slices. In contrast, total oxidation of propionic acid was comparable in both tissues. These results have led to a description of a new proposed metabolic pathway for fat synthesis in adipose tissue which includes propionate as a precursor(2). The present work was undertaken to determine whether this function of propionic acid in adipose tissue can be demonstrated in the intact animal, and whether this function is more pronounced in obesity.

*Methods.* Two groups of mice of the White Swiss strain were used: normal animals and animals made obese by injection of goldthioglucose(3). Both groups were fed *ad lib.* until time of experiment. Animals were injected intraperitoneally with 10 microcuries sodium propionate-1-C-14, in isotonic NaCl and were placed in all-glass, temperature-controlled metabolism cages with access to only water. $CO_2$ and urine were collected until time of sacrifice. Groups of animals (Table II) were killed by etherization at intervals up to 24 hours. The entire animal was immersed in alcoholic potassium hydroxide and heated overnight on a steam bath. Glycogen, fatty acids and non-saponifiable lipids were assayed

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TABLE I. Body Composition

| Mouse | Total wt, g | Lean wt, g | g lipids/g lean wt (× 100) |
|---|---|---|---|
| Normal (24)* | 31.4 ± .7† | 28.7 ± .6 | 9.4 ± .9 |
| Obese (16) | 44.3 ±1.7 | 35.8 ±1.4 | 24.4 ±2.9 |

* No. of animals. † Stand. error of mean.

for total amount and radioactivity as described previously(4).

*Results.* Comparison of body composition of the 2 experimental groups is shown in Table I. Obese mice averaged 40% higher in total weight than the normal. Although this weight gain was due in part to increase in tissue other than adipose, the greatest weight gain was at the expense of deposition of fat.

Table II shows a balance study of the C-14 from injected propionate. Values in this Table are averages of from 3 to 10 animals/group. Total recovery of administered propionate-1-C-14 varied from 87 to 105%. The major part of radioactivity administered was collected as expired $CO_2$. Glycogen and non-saponifiable lipids accounted for only negligible amounts of activity. Radioactivity recovered in urine and feces ranged from 0.2% at early intervals for individual mice to as much as 14% after 24 hours of collection.

A pronounced difference in conversion of propionate into lipids of obese and normal mice was noted. Five hours following injection, C-14 recovered as fatty acids in obese mice was 10.4% of administered activity compared to a value of 2.3% in the normal group. At 16 and 24 hours, there was less difference between the 2 groups: 4% for obese and 3.2% for normal at the former time, and 2.6% for obese and 2.0% for normal at the latter time.

These differences in fatty acid synthesis are shown graphically in Fig. 1. The data are expressed as lipid incorporation/gram lean body weight which more adequately represents metabolizing tissue for each group of animals (Table I). The curve for obese mice reaches a peak value at an early period and obtains a value 3 times greater than obtained by the normal group. This activity falls off rapidly

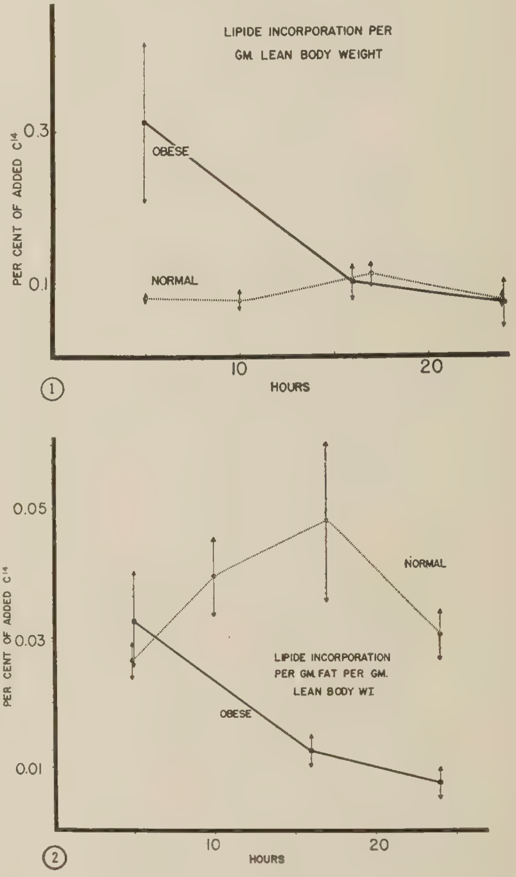

FIG. 1 and 2.

TABLE II. Metabolism of Propionate-1-C-14 Balance Study. (10 μcuries of sodium propionate-1-C-14 in isotonic NaCl was inj. intraper. into each mouse. Total activity assayed as 4 × $10^6$ cpm. Specific activity was 1 millicurie/millimole.)

| Mouse | No. of animals | Interval after inj, hr | Avg C-14 recovered as — $CO_2$ | Glycogen | Urine & feces | Fatty acids | Total |
|---|---|---|---|---|---|---|---|
| | | | % | | | | |
| Normal | 10 | 5 | 95.4 | .6 | 1.9 | 2.3 | 100.2 |
| | 3 | 10 | 91.2 | .1 | 4.6 | 2.3 | 98.2 |
| | 7 | 17 | 92.2 | .1 | 8.0 | 3.2 | 103.5 |
| | 4 | 24 | 89.6 | .2 | 8.3 | 2.0 | 100.1 |
| Obese | 5 | 5 | 88.8 | 1.5 | 2.4 | 10.4 | 103.1 |
| | 5 | 16 | 93.2 | .5 | 3.9 | 4.0 | 101.6 |
| | 6 | 24 | 90.9 | .3 | 4.2 | 2.6 | 98.0 |

so that at 24 hours the 2 groups are at the same level. In spite of variations of weight as well as differences in degree of obesity achieved, the differences between the 2 curves are significant, as evidenced by the bar lines representing standard deviations of experimental points.

Fig. 2 shows a comparison of normal and obese activities expressed as lipid incorporation/g of fat/g of lean body weight. Here the normal curve rises to a higher value than the curve obtained from obese animal primarily because of smaller stores of fat/unit animal in the normal group.

*Discussion.* That slices of adipose tissue converted propionate to fatty acids at rates comparable to acetate conversion while liver was relatively inactive, led to the suggestion that propionate might be selectively synthesized into newly formed fatty acids by adipose tissue in the intact animal. By using the C-14 labelled compound, it was thought a measure of amount of adipose tissue could be made with a balance study. This should yield a measure of the degree of obesity by employing a metabolic criterion.

Our studies indicate that the aims described above might be achieved. By assaying expired $CO_2$, excreta, lipids and glycogen, essentially 100% of administered radioactivity from propionate was recovered. Of radioactivity not eliminated as $CO_2$, urine and feces, 90% remained in the fatty acid fraction (see Table II) with glycogen making up the difference. Thus, the difference in amount of radioactive propionate injected and that collected in $CO_2$ and excreta gives an estimate of labelled fatty acids in the intact animal.

Propionate has been shown to be a precursor for body fat synthesis in the whole animal. In the obese animal, up to 10% of the C-14 remained as labelled fatty acids 5 hours after propionate-1-C-14 was injected. This value might be higher at earlier intervals.

Fall of radioactivity after the 5 hour peak indicates the rapidity by which body fat may turnover. Recent work by Dole and others (5) has shown that the unesterified fatty acid fraction in plasma has half-times of only a few minutes. These components of plasma are derived from adipose tissue and are insensitive to fat ingestion but are extremely sensitive to such physiological changes as are brought about by administration of glucose, insulin or epinephrin, and to other determinants of carbohydrate utilization. Fasting represents another such determinant. The animals were not allowed food during the experiment. Such mild starvation might have altered turnover of fat stores differently in each group of animals. Similar type experiments with controlled dietary intake are the subject of future investigation.

*Summary.* Twenty-four normal and 16 obese mice were injected with radioactive propionate and the tag was followed into $CO_2$, non-saponifiable lipids, fatty acids, glycogen, urine and feces. Propionate is a precursor for body fat synthesis in the whole animal. Conversion of propionate to lipids was greater in obese than in lean mice.

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# Thyroxine Secretion Rate of Mice.* (25333)

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The thyroxine secretion rate of mice was first determined by Hurst and Turner(1) using the goitrogen technic. Secretion rate in terms of D,L-thyroxine for various strains of female mice was as follows: $C_3H$ averaging 19.6 g, 2.2 μg; Schwing 19.9 g, 5.5 μg; and Rockland 32.7 g, 2.1 μg. This method is satisfactory for groups but cannot estimate individual thyroxine secretion rate. Amin *et al.* (2) applied a radioiodine thyroxine secretion rate technic to several strains of mice. The strain of mice used in our laboratory for study of mammary gland growth is described as "Swiss-Webster" obtained originally from Taconic Farms by R. F. Beyer and Son, Billings, Mo. In the extension of our studies to determine the role of thyroxine in mammary gland growth, it became necessary to determine the variation and mean thyroxine secretion rate (TSR) as a preliminary to replacement studies.

*Materials and methods.* Adult male and female mice (Beyer strain) were kept under conditions of uniform temperature (78 ± 1°F) in a room artificially illuminated during normal daylight hours and fed Purina Lab. chow. Each animal was injected i.p. with 3 μc carrier-free $I^{131}$. Maximum uptake was observed about 24 hrs later. Recycling of metabolized $I^{131}$ was prevented by feeding 0.01% tapazole in the ration. The procedure described for the rat(3) was followed except that increments of thyroxine were 0.5 μg/100 g body weight. The dose which prevented further thyroidal-$I^{131}$ output in each mouse (95-100% of previous count) was estimated as its TSR. Animals were gonadectomized 4 weeks previous to the determination of TSR.

*Results.* The mean TSR of the intact male and female mouse of this strain at the same age was identical, 1.2 μg/100 g body weight per day (Table I). In both the male and female, gonadectomy did not significantly reduce the TSR with the numbers included. In the female no significant change in TSR was noted with increasing age between 120 and 230 days of age. The mean TSR of 66 female mice was 1.25 ± 0.05 μg/100 g body weight with a range between 0.5 and 2.0 μg.

*Discussion.* Only a single previous study of thyroxine secretion rate in the mouse has appeared(2) in which the estimation was based on $I^{131}$ method. The TSR of inbred strains of male and female were as follows: A/Jax 2.13 ± 0.12 μg and 1.84 ± 0.10; BALB/c 2.44 ± 0.21 μg and 1.84 ± 0.12; C57BR/cd 3.35 ± 0.34 μg and 2.45 ± 0.11 and C57BL/6 4.10 ± 0.64 μg and 3.33 ± 0.34. The TSR of the males of each strain was consistently, but not significantly, higher than the female. In the present study no sex difference was noted in the Beyer strain. The mean TSR of the females (1.25 ± 0.05 μg) is lower than any of the inbred strains and in 2 cases only ½ as high.

The range in TSR of all age groups of female mice is of especial significance (0.5 to 2.0 μg/100 g body weight). Optimal levels of thyroxine for mammary gland growth can now be estimated in relation to the highest observed TSR. It is suggested that part of the variation in the extent of mammary gland growth in mice may be due to low TSR of individual mice. By the administration of thyroxine, equal to or above the highest level observed, deficiencies of thyroxine in relation to mammary gland growth may be eliminated.

*Summary.* The mean thyroxine secretion rate (TSR) of "Swiss-Webster" intact male and female mice at the same age (120 days) was identical, 1.2 μg/100 g body weight per

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TABLE I. Thyroxine Secretion Rate of Mice.

| | Group | Age, days | No. of mice | TSR ($\mu g$/100 g body wt) | Distribution of TSR .5 $\mu g$ | 1 $\mu g$ | 1.5 $\mu g$ | 2 $\mu g$ | 2.5 $\mu g$ |
|---|---|---|---|---|---|---|---|---|---|
| ♂ | Intact | 140 | 14 | 1.20 ± .15* | 3 | 6 | 3 | 1 | 1 |
| | Castrate | 140 | 12 | .83 ± .06 | 6 | 5 | | 1 | |
| ♀ | Intact | 120 | 26 | 1.26 ± .07 | | 16 | 6 | 4 | |
| | " | 140 | 10 | 1.20 ± .11 | | 7 | 2 | 1 | |
| | " | 180 | 9 | 1.33 ± .14 | 1 | 2 | 5 | 1 | |
| | " | 230 | 13 | 1.19 ± .01 | 2 | 5 | 5 | 1 | |
| | " | All age groups | 66 | 1.25 ± .05 | 3 | 30 | 18 | 7 | |
| | Castrate | 180 | 7 | .92 ± .06 | 1 | 6 | | | |

* Mean ± S.E.

day. Gonadectomy produced a consistent, but not significant, reduction in TSR in both sexes. In the female no significant change in TSR was noted with increasing age between 120 and 230 days of age. The mean TSR of 66 female mice maintained at a temperature of 78 ± 1°F was 1.25 ± 0.05 $\mu g$ 1-thyroxine/100 g/day, with a range between 0.5 and 2 $\mu g$.

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# Preferential Concentration of $C^{14}$ in Posterior Pituitary Following Hydrocortisone $C^{14}$ Injection.* (25334)

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Preferential uptake of some hormones by the pituitary gland has been reported by several investigators. Riegel *et al.*(1) have found the concentration of $C^{14}$ progesterone to be much higher in the pituitary and adrenals of rats than in plasma or other tissues. Joliot *et al.*(2) and other investigators(3-5) noted a preferential accumulation of thyroxine $I^{131}$ in the posterior pituitary. Courrier and Zizine (6) observed that 2 hours after administration of $C^{14}$ cortisone to rabbits, the kidneys and posterior pituitary had slightly higher tissue plasma ratios than other organs. In view of the great interest in the regulation of steroid hormone levels by the pituitary and hypothalamus, we undertook to confirm and extend these observations.

*Methods.* Hydrocortisone 4-$C^{14}$ (1.47 $\mu c$/mg) was injected intravenously into 4 rats, 5 guinea pigs and 2 rabbits. The amounts of hydrocortisone injected approximately doubled the hormone content in the body.§ The animals were anesthetized with ether, and the hormone in 1 ml of aqueous alcohol was injected into the foot vein. After 15 to 30 minutes, the vena cava was severed and the animal perfused through the aorta with 0.9% sodium chloride solution. The tissues were then excised, weighed, homogenized in 75% methanol and centrifuged. Precise separation

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§ Hydrocortisone is the normal adrenal secretory product of the guinea pig, while the rat and rabbit primarily secrete corticosterone(7). Apparently, corticosterone and hydrocortisone are handled similarly by the rat(8).

TABLE I. Distribution of $C^{14}$ after Hydrocortisone $C^{14}$ Injection.*

| | Posterior pituitary | | Anterior pituitary | | Liver | | Kidney | | Adrenal | | Muscle§ | | Brain‖ | | Thyroid¶ | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | cpm/mg | t/p† | cpm/mg | t/p | cpm/mg | t/p | cpm/mg | t/p | cpm/mg | t/p | cpm/mg | t/p | cpm/mg | t/p | cpm/mg | t/p |
| Rat | 8.5 ± 1.1‡ | 8.6 | | | 3.0 | 3.3 | 1.0 | 1.1 | 1.5 | 1.6 | .50 | .5 | .61 | .6 | | |
| ” | 21 ± 9.0 | 58 | 2.0 ±1.3 | 5.8 | .95 | 2.6 | .65 | 1.8 | 2.0 | 5.8 | .48 | 1.3 | .67 | 1.5 | | |
| ” | 2.7 ± 2.5 | 4.5 | .36 ± .32 | .6 | .41 | .7 | 1.3 | 2.0 | | | | | .05 | .07 | | |
| Adrex rat | 4.4 ± 2.2 | 14 | 1.8 ± .50 | 5.1 | 1.4 | 4.2 | 1.3 | 3.9 | | | | | .12 | .3 | | |
| Guinea pig | 3.7 ± 2.2 | 16 | .8 ± .8 | 4.2 | .58 | 2.6 | 1.5 | 6.7 | .46 | 2.0 | .25 | 1.0 | .78 | 3.5 | | |
| ” | 341 ±21.3 | 555 | 15.3 ±1.4 | 25 | .80 | 1.3 | 1.0 | 1.6 | .50 | .9 | .50 | .8 | .70 | 1.1 | 2.4 | 3.8 |
| ” | 1.9 ± 1.3 | 6.6 | .69 ± .45 | 2.2 | .77 | 2.7 | .73 | 2.5 | .49 | 1.9 | .26 | .9 | .09 | .32 | .16 | .6 |
| ” | 1.6 ± 1.1 | 4.0 | .33 ± .39 | .9 | .86 | 2.6 | 1.3 | 4.0 | 1.2 | 3.6 | .11 | .3 | .56 | 1.6 | .25 | .8 |
| ” | 21 ± 1 | 2.2 | .66 ± .42 | .7 | 2.6 | 2.8 | 2.0 | 2.2 | 1.5 | 1.6 | .40 | .5 | .72 | .7 | .70 | .8 |
| Rabbit | 2.8 ± .40 | 4.6 | | | .87 | 1.4 | 1.4 | 2.0 | .21 | .35 | .25 | .4 | .15 | .2 | | |
| ” | 38.5 ± 3.0 | 39 | 1.8 ± .23 | 1.7 | 2.1 | 2.1 | 2.3 | 2.3 | .39 | .39 | .80 | .8 | .18 | .1 | 1.0 | 1.0 |

* Radioactivity in methanol soluble fraction 30 min. after intrav. inj. of hydrocortisone 4-$C^{14}$. Dose: rats and guinea pigs were inj. with .16-.25 mg; rabbits, 1.2 mg.

† Tissue plasma ratio.

‡ Represents 99% error $= \pm 2.58 \sqrt{\left(\frac{N_s}{T_s}\right)^2 + \left(\frac{N_b}{T_b}\right)^2}$.

N and T represent numbers of counts and time of counting for samples and blanks respectively.

§ Heart and skeletal muscle showed no difference.

‖ Anterior hypothalamus, posterior hypothalamus, cortex, and cerebellum showed no difference.

¶ Activities of spleen, lung, thymus, and testis were approximately of the same order of magnitude and intermediate between that of adrenal and muscle.

of the hypothalamus, median eminence and the pituitary lobes was not always possible.|| The supernatant solution, or in the case of large organs, extracts from 100 to 200 mg of tissue were evaporated to dryness. In 3 instances, the residue was fractionated between 75% methanol and 4 volumes of chloroform to separate the free steroids (chloroform soluble) from the conjugated compounds (soluble in aqueous methanol). After the solvents were evaporated, the residues were extracted with 1 ml absolute ethanol. Plasma was deproteinized with 3 volumes methanol, and aliquots of the supernatant were used for $C^{14}$ assay. For radioactivity assay, the alcohol extracts were evaporated on 1.5″ flat aluminum discs and assayed with a plastic end window gas flow Geiger tube (efficiency about 600,000 cts/min/$\mu$c $C^{14}$). The weight of material on the discs was less than 1 mg and no self absorption corrections were used. In most cases, background counts were determined for each individual disc.

*Results.* We have observed, in agreement with others(8-10), that 2 minutes after injection only 20 to 30% of the injected counts could be accounted for in the plasma. Apparently the hormone is rapidly distributed throughout the body for within 15 to 30 minutes, radioactivity was found in all tissues examined (Table I). Results are presented as cts/min/mg of tissue and tissue/plasma radioactivity concentration ratio. In the case of the pituitary lobes, the 99% counting error has also been given. The 99% error equals 2.58 times the standard error and indicates that in 99 out of 100 determinations the values would fall within the indicated range. In general, liver and kidney had greater activity than muscle, brain, thyroid, spleen, lung, testis, and thymus. The striking observation is the high concentration of activity in the posterior pituitary. In 3 of 11 experiments, the activity (cts/min/mg) was more than 10 times that of the other tissues and in 6 experiments was more than 3 times the activity of the other tissues. In 2 experiments, however, the "specific activity" of the posterior pituitary was of the same order of magnitude as kidney or liver. In all cases, the posterior pituitary had at least 3 times the activity of the anterior lobe. In a single experiment, Courrier and Zizine(6) observed the highest activity in the kidney and the next highest in the posterior pituitary.

Since the posterior pituitary was the smallest organ counted, the absolute number of counts in the gland was often small. We, therefore, felt it advisable to use the 99% error rather than the more commonly used standard error.

*Discussion.* These experiments were performed in an attempt to determine whether receptor sites for circulating adrenal corticoids were present in hypothalamic or pituitary regions. Our results are compatible with the hypothesis that the posterior pituitary is relatively richer in such sites. Several regions of the hypothalamus were tested and no preferential concentration was observed.

The amount of $C^{14}$ found in the posterior pituitary is of the order of magnitude of 0.003-0.1% of the injected dose. Despite this variability, our results indicate a preferential accumulation of $C^{14}$ by the posterior pituitary of rats, rabbits, and guinea pigs following administration of hydrocortisone 4-$C^{14}$. However, it is not known what fraction of the $C^{14}$ bound in the posterior pituitary is unaltered hydrocortisone. The turnover of the hormone is known to be rapid and a substantial portion of the $C^{14}$ in the body may no longer be present as hydrocortisone. In 3 experiments, the $C^{14}$ activity was separated into a water soluble and chloroform soluble fraction (presumably conjugated and free steroids). In 2 of these, the aqueous extract contained less than 15% of the total tissue activity, while in one case about 50% was in the aqueous phase. Both fractions were preferentially concentrated in the posterior pituitary. Due to the low specific activity and limited amount of hormone, an identification of radioactive compounds was not attempted. To evaluate the physiological significance of our findings, further clarification of the nature of the activity in the pituitary will be essential.

*Summary.* Hydrocortisone 4-$C^{14}$ was in-

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|| Anterior lobe weights were: rat, 4.6-8.6 mg; guinea pig, 8.0-16.6 mg; rabbit, 9.1-17 mg. Posterior lobe weights: rats, 1.1-4.0 mg; guinea pig, 2.0-4.7 mg; rabbit, 3.2-6.0 mg.

jected into rats, guinea pigs, and rabbits, and distribution of $C^{14}$, 30 minutes after injection was studied. Activity was widely distributed and the results were variable; but in most experiments, activity (cts/min/mg tissue) was higher in the posterior pituitary gland than in any other tissue. Activity in the anterior pituitary, liver, and kidney was higher than that in muscle and brain.

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## Use of Human Kidney Cultures in the Study of Enteroviruses.* (25335)

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Use of human kidney tissue in suspended cell cultures for propagation of polioviruses was reported by Weller *et al.*(1); however, studies on Coxsackie and ECHO viruses have been conducted almost entirely in other tissue culture systems, chiefly rhesus monkey kidney. It was previously reported that kidney tissue cultures from relatively few monkey species have proven satisfactory for studies of the entire family of enteroviruses(2,3), and cultures prepared from non-primate kidney tissues have consistently proved unsatisfactory for propagation of these viruses(4). For these reasons the susceptibility spectrum of primary human kidney cell cultures to the 3 groups of enteroviruses (poliomyelitis, Coxsackie and ECHO viruses) was investigated.

*Materials and methods. Source of material.* Human kidney specimens were obtained following surgical removal or at autopsy. In the latter case only those kidneys were used which had been removed within a few hours of death, with the capsule intact and, in so far as possible, with aseptic precautions, to avoid gross contamination. After examination by the pathologist, kidneys were delivered to our tissue culture laboratory in a sterile jar which contained Hanks balanced salt solution and antibiotics. *Preparation of human kidney (HK) cultures.* The human kidney tissues were trypsinized in the same manner used for preparation of monkey kidney cultures(5). The trypsinized cell suspensions were centrifuged and the packed cells resuspended in growth medium (Hanks salt solution, 0.5% lactalbumin hydrolysate, 10% medium #199, and 20% calf serum) at a final cell concentration of 3-5 x $10^5$ cells/ml. Three-ounce prescription bottles and tubes (16 x 150 mm) were seeded with 10 ml and 1 ml, respectively and incubated at 37°C. When a confluent cell sheet was obtained, usually within 3-5 days, the growth medium was removed and replaced with maintenance medium containing Earles salt solution, 0.5% lactalbumin hydrolysate, and 2% calf serum. In cases where cell growth appeared to be poor, the cultures were usually changed with fresh growth medium 3-5 days after seeding and

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TABLE I. Cell Growth of Human Kidney Cultures.

| Age (yr) | Specimens obtained hr after: Surgery | Death | No. grown /No. cases | % showing growth |
|---|---|---|---|---|
| Adult (37–76) | <2 | | 5/5 | 100 |
| " (28–87) | | 3– 6 | 4/6 | 67 |
| | | 10–24 | 2/17 | 12 |
| Baby (0–2) | | 3– 6 | 5/6 | 84 |
| | | 8–24 | 32/38 | 84 |
| | | >24 | 0/2 | 0 |

incubated until a confluent cell sheet was obtained. *Virus strains.* Poliovirus, types 1, 2, 3, (virulent and attenuated strains), Coxsackie virus, types A-9, B 1-5 and ECHO virus types 1-14, were studied. Stock strains were all derived from rhesus monkey kidney cultures. Stool samples, as a source of virus, were obtained from apparently healthy individuals as well as from those suffering from a variety of febrile illnesses. Mouse brain suspension containing known Coxsackie A-9 virus was used.

*Results. Growth of HK cultures.* Table I summarizes experiences with growth of cultures prepared from human kidney tissue of 74 individuals. The cells from kidneys removed at operation grew in cultures with considerable ease. Success was attained in growing 4 out of 6 (67%) autopsy specimens from adults obtained less than 6 hours after death; whereas, only 2 of 17 (12%) kidney tissues were grown when the kidney was removed more than 10 hours after death. On the other hand, kidney tissues from babies, especially premature babies, yielded good cell cultures, even though the specimens were collected 10-24 hours after death. All cultures, either derived from adult or from baby kidney cells, were kept in satisfactory condition for as long as 2 months when the cultures were changed with maintenance medium weekly. In no instance were latent viruses observed.

*Comparative susceptibility of rhesus (Rh) and HK cultures to enteroviruses.* Poliovirus types 1, 2, and 3 (both virulent and attenuated strains) produced marked cellular degeneration in adult and baby HK cultures and in Rh cells 18 hours after inoculations (Table II). Plaque titers as well as virus yields were almost the same in both cell systems (Table II). Baby HK cultures were highly sensitive to Coxsackie and ECHO viruses and displayed distinct cytopathogenic changes (Figs. 1 and 2), similar to those produced by polioviruses. Virus yields in baby HK cultures were as good or better than those produced in Rh cells. However, certain adult HK cultures did not show similar responses when inoculated with Coxsackie A 9, Coxsackie B 1-5 or ECHO viruses (except ECHO virus types 7 and 10). Plaque titers were lower in adult HK cultures than in Rh cultures inoculated with these viruses (Table II). Incomplete cellular destruc-

TABLE II. Cytopathogenic Effect and Virus Titers of Poliomyelitis, Coxsackie and ECHO Viruses in Rhesus Monkey and Human Kidney Cultures.

| Virus | CPE at time indicated after infection: Rh | HK (B) | HK (A) | Virus titer log (PFU/ml) measured in cultures of: Rh | HK (A) | Virus yields (PFU/ml) in cultures of: Rh | HK (B) |
|---|---|---|---|---|---|---|---|
| | (18 hr) | (18 hr) | (18 hr) | | | | |
| Poliovirus type 1 (also types 2 & 3) | +++ | +++ | +++ | 7.8 | 7.8 | 7.7 | 7.8 |
| | (2 days) | (2 days) | (14 days) | | | | |
| Coxsackie Group A-9 (also Group B 1-5) | +++ | +++ | ± | 7.7 | 6.0 | 7.5 | 7.9 |
| ECHO type 1 (also types 2,3,4,5,6,8, 9,11,12,13,14) | +++ | +++ | ± | 7.6 | 5.8 | 7.5 | 7.3 |
| ECHO type 7 | +++ | +++ | ++++ | 6.7 | 6.6 | 7.8 | 7.1 |
| " " 10 | + | +++ | ++++ | N.D. | N.D. | N.D. | N.D. |

Rh = Rhesus monkey kidney cultures. HK (A) = Human (adult) kidney cultures. HK (B) = human (baby) kidney cultures.

+, A few cells degenerated. +++, ¾ of the cell sheet degenerated. ++++, Complete degeneration. ±, Questionable. N.D., Not done.

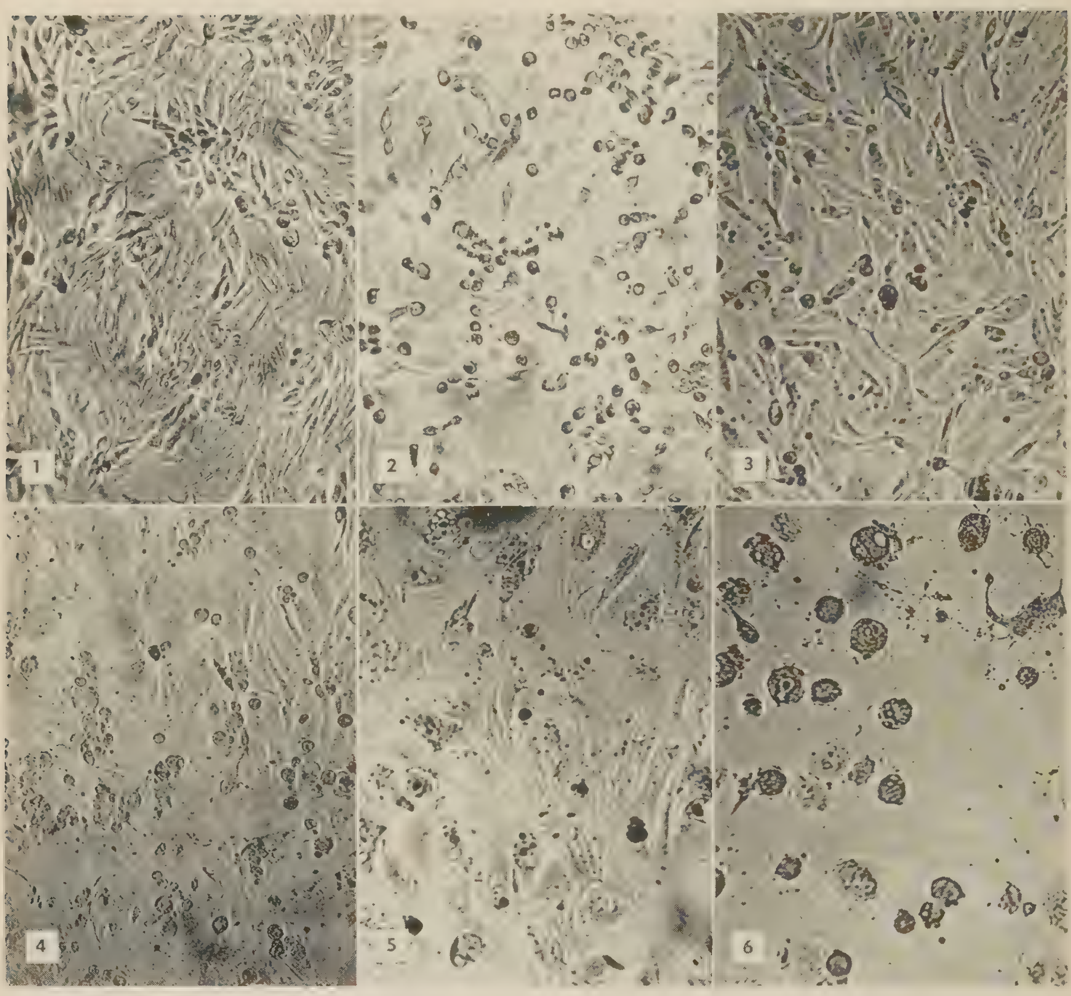

FIG. 1. Human (baby) culture control (7 days old).

FIG. 2. Human (baby) kidney culture infected with Coxsackie A9 virus, 0.1 ml of undiluted tissue culture fluid (18 hr after infection).

FIG. 3. Human (adult) kidney culture control (7 days old).

FIG. 4. Human (adult) kidney culture infected with Coxsackie A9 virus, 0.1 ml of undiluted tissue culture fluid (18 hr after infection).

FIG. 5. Human (adult) kidney culture infected with Coxsackie A9 virus (same sample as in Fig. 4 except 25 days after infection).

FIG. 6. Human (adult) kidney culture infected with Coxsackie A9 virus (as shown in Fig. 5) challenged with poliovirus type 1 (24 hr after challenge).

tion was observed 1-2 days after infection (Figs. 3 and 4) and then the cultures gradually recovered, although the viruses persisted in the fluid as long as 14-27 days. When an adult HK culture infected with Coxsackie A 9 virus 25 days previously (Fig. 5) was challenged with type 1 poliovirus, complete cellular destruction occurred within 24 hours (Fig. 6). Certain other enteroviruses, for example Coxsackie B5, which like Coxsackie A9, gave only limited degeneration in adult HK cultures, did, however, interfere with multiplication of subsequently inoculated poliovirus. Details of these interference experiments will be published.

*Isolation of enteroviruses in HK cultures.* Sensitivity of rhesus and human kidney cultures for primary isolation of enteroviruses directly from human stool or from mouse brain suspensions was also compared. Examples of such isolations are illustrated in Table III. Coxsackie A9 virus was readily isolated from mouse brain suspension in both Rh and baby HK cultures but not in adult HK cells. Rhe-

TABLE III. Isolation of Coxsackie and ECHO Viruses in Rhesus Monkey and Human Kidney Cultures.

| Material | Days after inoc. | CPE at different virus dilutions | | | | | | | | | Virus identified |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Rh | | | HK (A) | | | HK (B) | | | |
| | | Undil. | $10^{-1}$ | $10^{-2}$ | Undil. | $10^{-1}$ | $10^{-2}$ | Undil. | $10^{-1}$ | $10^{-2}$ | |
| Mouse brain suspension | 4 | +++ | ++ | ++ | 0 | 0 | 0 | 0 | 0 | 0 | Coxsackie A-9 |
| | 10 | | | | 0 | 0 | 0 | +++ | + | 0 | |
| Stool #C suspension | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | ECHO-2 |
| | 10 | +++ | +++ | + | 0 | 0 | 0 | +++ | ++ | + | |
| Stool #WB suspension | 4 | 0 | 0 | 0 | 0 | 0 | 0 | + | 0 | 0 | " -11 |
| | 10 | ± | 0 | 0 | 0 | 0 | 0 | +++ | +++ | ++ | |

See Table II for legend.

sus monkey kidney cultures and baby HK cultures were equally satisfactory for isolation of ECHO virus type 2 from stool suspensions. The baby HK cultures were highly sensitive for isolation of ECHO-11 (WB) virus from stool suspensions, whereas Rh cells were less susceptible to infection by this strain of virus. From 19 stool samples 8 (42%) yielded ECHO-11 (WB) virus when inoculated into baby HK cultures but ECHO-11 (WB) was isolated from only 3 (16%) when these 19 specimens were inoculated into rhesus monkey kidney cells. Furthermore, the 3 positive results with ECHO-11 virus obtained in rhesus cultures were also positive in baby HK cultures and were achieved only on stool samples containing high titers of virus. In no instance did adult HK cultures show distinct cytopathic changes when inoculated with these samples.

*Discussion.* Human kidney tissue obtained at autopsy from individuals of all ages usually are suitable for preparation of cultures if the specimens are processed within 6 hours after death. Difficulties encountered in prompt collection of specimens often limit the number of samples available. Consequently, there is a shortage of tissue suitable for preparing cultures.

The reason for the differences in susceptibility of adult and baby HK cultures to Coxsackie and ECHO viruses is obscure. This difference was not observed with polioviruses, but a similar phenomenon was noted by Henderson and Taylor(6) in studies of sandfly fever viruses in tissue culture. Our observation of the differences in sensitivity of kidney cells from young and mature individuals suggests that these differences may be the result of a type of cellular immunity present in tissues from adults. Dalldorf *et al.*(7) found that suckling mice were highly susceptible to Coxsackie viruses but adult mice were resistant to this group of agents. Recently McLaren and Sanders(8) have shown that this influence of age on susceptibility to Coxsackie viruses is due to the "accessibility" of host cells to the virus. In view of this, it seems possible that kidney cells from infants may be more accessible to Coxsackie and ECHO viruses than kidney cells obtained from older individuals.

The advantage of using baby HK cultures for isolation of ECHO viruses, for example ECHO-11 (WB) virus, is that these cultures apparently are more sensitive than monkey kidney cells, and, therefore, may facilitate detection of some of the elusive viruses of human infections.

*Summary.* 1) Human kidney cultures were prepared successfully from tissues obtained following surgical removal or at autopsy from individuals of all ages, when specimens were collected promptly. 2) Baby kidney cells were superior to adult both for culture growth and for isolation and propagation of enteroviruses. 3) Poliovirus types 1, 2 and 3 (virulent and attenuated strains) grew well in both adult and baby HK cultures. In contrast, Coxsackie A9, B 1-5 and all ECHO viruses tested (except types 7 and 10) induced distinct cellular degeneration only in baby HK cultures, while incomplete destruction with low virus titers were obtained in cultures derived from certain adult human kidneys. 4) The advantages of using baby HK cultures for isolation of certain ECHO viruses are discussed.

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## Zone Electrophoretic Studies of Proteins and Glycoproteins of Bovine Serum and Synovial Fluid. (25336)

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(Introduced by G. W. James III)
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The electrophoretic patterns of bovine serum and synovial fluid were determined as part of a broader study in the human. The scarcity of information concerning bovine synovial fluid glycoproteins prompted this report.

*Methods.* Undialyzed samples of serum or synovial fluid were subjected to electrophoresis at room temperature, on Whatman 3 mm paper, 3 cm. wide, in barbital buffer of ionic strength 0.075, at pH 8.6, for 16 hours at a constant current of 0.6 milliampere/strip. The Spinco model R series C, paper electrophoresis apparatus was used. After oven drying at 110°C, for 15 minutes, glycoproteins were additionally fixed to the papers and cleared of buffer salts by a wash in absolute alcohol. Paper strips were dyed for proteins with amidoschwarz 10 B dye and for glycoproteins by a modification of the periodic acid-Schiff technic(1). The dyed paper strips were analyzed in a Spinco model RA densitometer, using Wratten filters No. 58 and 22 for proteins and an interference filter with maximal transmission at 561 mu for glycoproteins. Synovial fluids were digested by 140 turbidity reducing units of bull testicular hyaluronidase at 37°C for 1½ hours prior to electrophoresis to remove the viscous anomaly. Weight of protein applied to each paper strip was equivalent to 10 microliters of a 4.5 g/100 ml solution of protein. For glycoprotein determinations 30 microliters of serum and 50 microliters of synovial fluid were applied to the paper. Protein concentration was determined by biuret method and protein-bound hexose by modification of the anthrone tryptophane reaction(2).

*Material.* Serum from 13 cows was obtained immediately after slaughter. Synovial fluid was aspirated from third joint of foreleg at the same time. In eight animals bilateral joints were aspirated making a total of 21 specimens of synovial fluid. This material was obtained through the courtesy of Hormel Co., Austin, Minn.

*Results.* Table I describes the mean protein and glycoprotein patterns of 13 bovine sera. Mean values from 13 human control sera are included for comparison. There was

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TABLE I.

| | Protein, g/100 ml | % of total protein | | | | Protein, g/100 ml | | | | Hexose, mg/100 ml | % of total protein-bound polysaccharide | | | | Hexose/protein (PR) ratio |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Alb. | $(\alpha_1+\alpha_2)$ | $\beta$ | $\gamma$ | Alb. | $(\alpha_1+\alpha_2)$ | $\beta$ | $\gamma$ | | Alb. | $(\alpha_1+\alpha_2)$ | $\beta$ | $\gamma$ | |
| | | | | | | Normal human serum | | | | | | | | | |
| Mean | 7.09 | 53 | 7 11 | 14 | 17 | 3.72 | .47 .75 | .96 | 1.18 | 100 | 13 | 16 25 | 25 | 20 | 1.42 |
| S.D. | .27 | 3 | 1 2 | 1 | 2 | .37 | .05 .06 | .07 | .20 | 8 | 3 | 4 5 | 4 | 3 | .12 |
| | | | | | | Normal bovine serum | | | | | | | | | |
| Mean | 8.08 | 42 | 15 | 15 | 28 | 3.37 | 1.19 | 1.23 | 2.28 | 124 | 7 | 51 | 23 | 20 | 1.53 |
| S.D. | .64 | 3 | 2 | 2 | 4 | .29 | .10 | .15 | .49 | 12 | 4 | 6 | 4 | 5 | .12 |
| | | | | | | Normal bovine synovial fluid | | | | | | | | | |
| Mean | 1.80 | 50 | 14 | 16 | 21 | .90 | .24 | .29 | .37 | 36 | 10 | 49 | 28 | 14 | 1.98 |
| S.D. | .66 | 5 | 2 | 2 | 3 | .27 | .08 | .10 | .11 | 11 | 5 | 8 | 5 | 4 | .46 |

8.08 ± .64 g of protein and 124 ± 12 mg of protein-bound hexose/100 ml of bovine serum. Alpha 1 and alpha 2 globulins were only infrequently separated by the conditions of electrophoresis used. There was 3.37 ± .29 g of albumin, 1.19 ± .10 g of alpha globulin, 1.23 ± .15 g of beta globulin and 2.28 ± .49 g of gamma globulin/100 ml of bovine serum. In comparison to human serum, bovine serum contained significantly more beta and gamma globulin, significantly less albumin and essentially the same concentration of alpha globulins. The marked increase in gamma globulin accounted for the higher bovine serum protein.

The protein-bound hexose of bovine serum (124) was significantly higher than human serum (100) but the mean bovine hexose/protein (PR) ratio of 1.53 ± .12 did not differ significantly from the mean human value of 1.42 ± .12. The distribution of protein-bound polysaccharide in bovine serum was similar to human serum with slightly less albumin polysaccharide and slightly more alpha globulin polysaccharide.

Table I also describes the mean protein and glycoprotein patterns of 21 bovine synovial fluids. There was 1.80 ± .66 g of protein and 36 ± 11 mg of protein-bound hexose/100 ml of bovine synovial fluid. The protein concentration of synovial fluid was only 22% of the serum protein, but the synovial fluid had a higher relative concentration of albumin (50%) and a lower relative concentration of gamma globulin (21%) than the comparable fractions in serum (albumin—42%, gamma globulin 28%). The distribution of bovine synovial glycoproteins was essentially comparable to serum glycoproteins but the failure to distinguish the alpha 1 and alpha 2 zones may have masked significant differences between fluid and serum.

The mean hexose/protein (PR) ratio of bovine synovial fluid (1.98 ± .46) was significantly higher than bovine serum (1.53 ± .12) indicating preferential permeability of one or more glycoproteins.

*Comment.* The electrophoretic pattern of bovine serum (or plasma) has been studied in a few animals by moving boundary electrophoresis using a variety of buffers(3-5). Considerable variation in these reports resulted from differences in technic, with albumin ranging from 38-43% and gamma globulin from 11 to 28%. In a large series of 51 Devon steers, Brandish *et al.*(6), using moving boundary electrophoresis in a phosphate buffer of pH 7.5 and an ionic strength 0.18, noted albumin to comprise 46.6%, alpha globulins 14%, beta globulins 8.9% and gamma globulins 30.5% of a mean serum protein of 7.0 ± .5/100 ml. They noted that albumin remained relatively stable in their normal bovine population with a mean concentration of 3.2 g/100 ml while total serum protein varied with changes in gamma globulin. The thirteen bovine sera studied, in the present report, had an albumin concentration of 3.37 g per 100 ml with a higher total protein (8.08) which related to an increase in gamma globulin.

Hesselvick(7), using moving boundary electrophoresis, demonstrated hyaluronic acid, albumin, beta and gamma globulins in equine synovial fluid. Subsequent investigators(8) have shown alpha globulins to be present in bovine synovial fluid and have measured higher relative concentrations of albumin and lower relative concentrations of globulin in synovial fluid in comparison to serum. The results of the present report are in accord with these findings.

Neuhaus *et al.*(9) eluted the alpha, beta and gamma globulins of bovine synovial fluid after electrophoresis on starch and measured the protein and hexosamine concentration of each fraction, noting an increase in the hexosamine/protein ratio of the fluid in comparison to serum. The increase in the hexosamine/protein ratio was predominantly in the alpha globulin zone and was considered to result from the preferential permeability of seromucoid. The present report supports the finding of a relative increase in the glycoproteins of synovial fluid as compared to serum but does not offer evidence as to the identity of this glycoprotein(s). Neuhaus(9), other(10), and the present authors were not able to readily separate the alpha 1 and alpha 2 globulins of bovine serum and synovial fluid.

*Summary.* 1) Zone electrophoresis of 13 bovine sera yielded a mean concentration of 3.37 ± .29 g (42%) albumin, 1.19 ± .10 g (15%) alpha globulin, 1.23 ± .15 g (15%) beta globulin and 2.28 ± .49 g (28%) gamma globulin/100 ml. Serum protein-bound hexose was 124 mg/100 ml or 1.53 ± .12% of the mean serum protein concentration of 8.08 g/100 ml. Zone electrophoretic separation showed albumin to contain 7 ± 4%, alpha globulins 51 ± 6%, beta globulins 23 ± 4% and gamma globulins 20 ± 5% of the protein-bound polysaccharide. 2) Twenty-one synovial fluids from the same animals had a mean concentration of .90 ± .27 g (50%) albumin, .24 ± .08 g (14%) alpha globulins, .29 ± .10 g (16%) beta globulins and .37 ± .11 g (21%) gamma globulin/100 ml. Synovial fluid protein-bound hexose was 36 ± 11 mg/100 ml or 1.98 ± .46% of the mean synovial fluid protein of 1.80 g/100 ml. Zone electrophoretic separation showed albumin to contain 10 ± 5%, alpha globulins 49 ± 8%, beta globulins 28 ± 5%, and gamma globulins 14 ± 4% of the protein-bound polysaccharide. 3) The synovial fluid had relatively more albumin (50%) and relatively less gamma globulin (21%) than serum (albumin 42%, gamma globulin 28%) and a higher hexose/protein (PR) ratio indicating preferential permeability of albumin and one or more glycoproteins. Alpha globulins were not separated into alpha 1 and alpha 2 zones.

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# Normal Growth of Rat Mammary Glands During Pregnancy and Lactation.* (25337)

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Previous studies of normal and experimental development of mammary gland have used morphological qualitative indices to measure extent of duct and lobule-alveolar growth. Kirkham and Turner(1) first suggested the use of desoxyribonucleic acid (DNA) as a quantitative index of mammary gland cellular proliferation in rats. More recently studies have been conducted on rats(2,3), rabbits(4) and mice(5,6,7). It has been shown that DNA/nucleus is constant during pregnancy but drops 42.8% during early lactation(8). With a quantitative estimate of mammary gland growth in individual animals available, it is possible to determine the mean and normal variation in total mammary proliferation in individual rats at end of pregnancy. These data will serve as a standard of comparison in study of extent of mammary gland development in experimental animals stimulated with estrogen and progesterone(2). It will be possible, also, to determine quantitatively the synergistic influence of growth hormone, thyroxine, glucocorticoids, etc., on normal mammary gland growth during pregnancy and lactation.

*Methods.* Data were obtained from primiparous Sprague-Dawley-Rolfsmeyer rats during late pregnancy (18-20 days) and on days 5, 10, 14 and 21 of lactation. Rats ovariectomized on day 2 of lactation and sacrificed on day 14 were included. Six abdominal-inguinal mammary glands were removed from each rat and placed in deep freeze for 4 days, extracted in 95% ethanol for 3-5 hours and in ether for an additional 3-5 hours. Dry, fat-free tissue (DFFT) was weighed and ground to a fine powder in a Wiley mill. DNA of a 25 mg aliquot of tissue was determined by the method of Webb and Levy(9). Product of quantity DNA/mg DFFT and DFFT/100 g body weight was estimated as "total DNA." Five animals were anesthetized with nembutal and all nipples were covered except the 3 right abdominal-inguinal glands. The animals were then injected with oxytocin and 6 young were allowed to nurse for a sufficient time to insure complete milk removal from untaped glands. This procedure allowed one to compare DNA of lactating glands with and without milk.

*Results.* In initial experiments, separate DNA determinations were made on 4 pectoral glands and 6 abdominal-inguinal glands. Sheets of muscular tissue located between pectoral glands were impossible to remove; therefore, only abdominal-inguinal glands, which are free of muscle, were used in estimating DNA content. There was no significant difference between mean DNA of pectoral glands (9.01 mg/100 g) and abdominal-inguinal glands (8.46 mg/100 g) of pregnant rats. Highly significant differences (P .001) exist between total DNA of late pregnancy and various stages of lactation (Table I). Total DNA on day 10 of lactation and day 14 in ovariectomized rats approached significance (P .05) as compared to day 5 of lactation. Statistically significant difference (P .01) was observed between glands containing milk and those without milk. Average total DNA in nursed glands was 5.81 mg/100 g (5.36-6.39); those with milk averaged 7.31 mg/100 g (6.52-8.54 mg/100 g). Dry, fat-free milk did not interfere with color reaction in determination of DNA.

*Discussion.* It has been considered that mammary gland growth occurs primarily during the first ⅔ of pregnancy, followed by gradual initiation of lactation during final trimester. It has not been possible to determine by morphological methods additional growth of alveoli during the latter part of pregnancy or lactation since secretion of colostrum and milk obliterates the individual alveoli. Re-

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TABLE I. Total Mammary Gland DNA during Late Pregnancy and Lactation.

| No. of animals | Stage of gestation or lactation | Body wt (g), mean | DFFT (mg/100 g body wt), mean | DNA ($\mu$g/mg DFFT), mean ± S.E. | Total DNA (mg/100 g), mean ± S.E. |
|---|---|---|---|---|---|
| 19 | 18-20 days of pregnancy | 324.3 | 230.9 | 33.2 ± .84 | 7.63 ± .40 |
| 10 | 5th day of lactation | 301.5 | 319.3 | 34.1 ± .73 | 10.8 ± .52* |
| 10 | 10th *Idem* | 302.2 | 390.8 | 31.8 ±1.2 | 12.26 ± .25* |
| 25 | 14th " | 298.1 | 417.5 | 26.3 ± .5 | 10.9 ± .39* |
| 9 | 21st " | 304.7 | 396.6 | 28.3 ± .45 | 10.31 ± .02* |
| 18 | Castrate, 2nd day; lactation, 14th day | 290.4 | 572.7 | 22.3 ± .4 | 12.75 ± .64* |

* Significant at .1% level when compared to pregnant level. DFFT = Dry, fat-free tissue.

cently Wada and Turner(7), using DNA as an index of growth, have indicated that in mouse a continued proliferation of epithelial cells occurs from day 12 to 18 of pregnancy. The present study is believed to indicate cellular proliferation during lactation on the basis of a 41.5% increase in DNA of lactating glands in comparison with those of late pregnancy. Growth appears to continue until day 5 or 10 of lactation and cellular replacement may occur until weaning since no reduction in total DNA was observed by day 21. A complicating factor in the measurement of proliferation during lactation is the 42.8% reduction in DNA/nucleus during lactation as compared to pregnancy values. Decrease in DNA/nucleus coupled with the 41.5% increase in total DNA during lactation would indicate a tremendous growth during this period. The difference between milked and unmilked glands indicates the possibility that some DNA component is present in milk. It has been demonstrated that desoxyribonuclease (DNA'ase) activity increases 100% on day 1 of lactation, while the acid-soluble components of supernatant decrease at this time(10). This observation would indicate possibility that enzymatic products of DNA may be involved in milk synthesis and lower estimation of total DNA in milked glands. Milk within gland does not mask the mammary proliferation during lactation since total DNA of the nursed glands when doubled equals values normally obtained on day 5 of lactation.

Rats normally ovulate shortly after parturition. If conception does not occur, corpora lutea of lactation form and persist until weaning. It was considered that ovarian hormones secreted by corpora lutea of lactation might be responsible for additional gland growth. However, the failure of mammary glands of ovariectomized lactating rats to return to pregnancy values indicates that gland growth in early lactation is not dependent upon the ovarian hormones. It has been suggested that lactogen(11) and growth hormone (12) have capacity for stimulating mammary gland growth. Recently, in this laboratory a lactogen-free anterior pituitary fraction (mammogen) has been extracted which is effective in producing lobule-alveolar growth in mice(13). It is suggested that mammary gland growth observed during early lactation may be due to the release of mammogen as a result of the nursing stimuli comparable to release of lactogen(14,15). This growth may be synergized by increased secretion of thyroxine(16), growth hormone, and other hormones secreted in increased amounts during lactation(17).

*Summary.* With DNA as a measure of mammary gland growth in rat, it was shown that there was a 41.5% increase between 18-20th day of pregnancy and day 5 of lactation. This increase occurred in spite of the fact that DNA/nucleus of lactating cells is reduced 42.8% in comparison with cells of pregnant animals. Increased total DNA for 6 abdominal-inguinal glands/100 g body weight was maintained until day 21 of lactation. Animals ovariectomized on day 2 of lactation showed equal increases on day 14 of lactation. It is concluded that marked glandular proliferation occurs following parturition (5 to 10 days) which is not interfered with by ovariectomy. It is suggested that post-parturient mammary gland growth, instead of depending upon ovarian hormones, may be due to direct

release of pituitary mammogen associated with release of oxytocin and lactogen by periodic nursing stimuli.

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## Glucagon Antibodies and Their Use for Immunoassay for Glucagon.* (25338)

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Clarification of the physiologic status of glucagon may well depend upon development of a practical and specific method for its detection in body fluids. Bioassay technics of "glucagon-like" activity have recently been developed(1,2,3) but are fraught with many serious objections(4). It seems that an immunoassay for glucagon, similar to those being developed for insulin(5,6,7), would provide greater specificity. The present report deals with efforts to develop an immunoassay for glucagon.

*Methods.* Female rabbits were given monthly subcutaneous injections of crystalline "insulin-free" beef-pork glucagon (Lilly†) suspended in Freund's adjuvant. A control group received either Freund's adjuvant alone, Freund's adjuvant plus insulin, or no injections at all. At intervals of several weeks specimens of serum were obtained and tested for development of non-precipitating antibodies to glucagon by means of Berson-Yalow radiochromatographic and electrophoretic technics used by these authors to detect insulin antibodies(8). The Berson-Yalow procedure(8) was adapted without major change, except that glucagon-$I^{131}$ was substituted for insulin-$I^{131}$. In general 0.01 $\mu$g of glucagon-$I^{131}$ was added to 1 cc of rabbit serum and incubated with shaking for 2 hours at 37° C. Ten to 50 $\mu$l were applied to cathodal end of Whatman No. 3 mm filter paper strip and chromatographed in barbital buffer at pH 8.6 for one hour. In some instances this was followed by 16 hours of electrophoresis. After heat drying for 1 hour at 120°C, staining with naphthalene blue black, and destaining in methyl alcohol and 10% acetic acid for 10 minutes, the strips were divided and segments counted in well-type scintillation counter or placed intact in an actigraph and counted with TGC-2 Geiger-Muller tube. In the latter case a count rate tracing was obtained with Texas Instruments Rectiriter Recorder. In addition the Skom-Talmadge technic for detection of non-precipitating insulin antibodies(9) by precipitation of radio-insulin-globulin complex with antiglobulin was adapted unchanged to study glucagon antibodies, except that glucagon-$I^{131}$ and anti-rabbit globulin were used. Glucagon-$I^{131}$ was iodinated by modification of Pressman-Eisen technic and yielded specific activities at time of use, from 13.5 to 14.1 μc/μg. By this method damage to the fragile glucagon molecule as determined by criteria of Berson, Yalow, and Volk(10) was restricted to 4-8% at time of use.

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† Supplied through generosity of Dr. W. R. Kirtley, Eli Lilly Co. Indianapolis, Ind., and considered to be very pure(4) with at most no more than 1% insulin contamination.

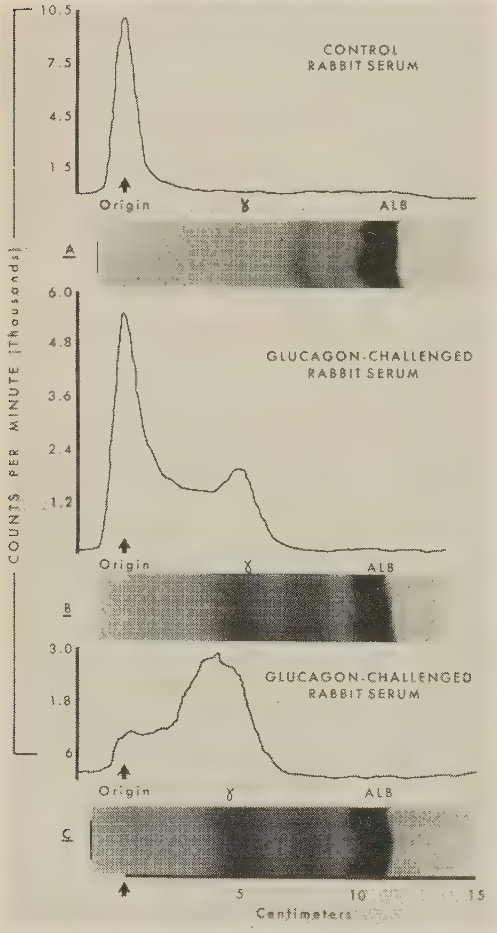

FIG. 1. A. Incubation of glucagon-$I^{131}$ with sera from all 3 control groups of rabbits characteristically resulted in above paper radioelectrophoretic pattern in which radioactivity is concentrated at origin of strip. B and C. Incubation of glucagon-$I^{131}$ with sera from glucagon-challenged rabbits gave distinctly contrasting radioelectrophoretic patterns, in which radioactivity migrates in varying degrees with the gamma globulin fraction. Note, too, the remarkable increase in gamma globulin which occurs in glucagon-immunized rabbits.

*Results. Control rabbits*: According to Berson, Yalow, and Volk(10) glucagon-$I^{131}$, like insulin-$I^{131}$, binds firmly to filter paper at point of application (origin). Fig. 1A demonstrates that when 0.1 μg of glucagon-$I^{131}$ is incubated with serum and subsequently subjected to paper electrophoresis most radioactivity remains at the origin. The small amount of radioactivity which migrates with serum proteins represents, according to Berson and co-workers(10), damaged glucagon-$I^{131}$. After its incubation with serum of rabbits from each of 3 control groups, the migration of 0.01 μg of glucagon-$I^{131}$ averaged 6.5% (4.8-8.8%), an average of 93.5% (91.2-95.2%) remaining bound to origin of strip.

*Glucagon-challenged rabbits.* Monthly injections of glucagon in Freund's adjuvant resulted in progressive change from above pattern (Figs. 1B, C). After incubation with serum from rabbits challenged with glucagon for 3 months or more, an average of 73.7% (45.9-87.3%) of radioactivity of 0.01 μg of glucagon-$I^{131}$ migrated with the gamma globulin fraction of the serum. An average of only 26.3% (12.7-54.1%) remained at the origin. This was assumed to be the consequence of binding of glucagon-$I^{131}$ to a non-precipitating antibody-like substance similar to the Berson-Yalow insulin antibody.

The Skom-Talmadge technic of precipitation of antigen-antibody complex with rabbit antiglobulin gave results which were virtually identical to the Berson-Yalow radiochromatographic technic.

Cross reactions between insulin-$I^{131}$ and glucagon antibodies or between glucagon-$I^{131}$ and insulin antibodies did not occur.

Kinetic studies patterned after those by which Berson and Yalow characterized the reaction between insulin and its antibody are in progress. Thus far, it appears that the glucagon-antibody complex is rapidly formed and

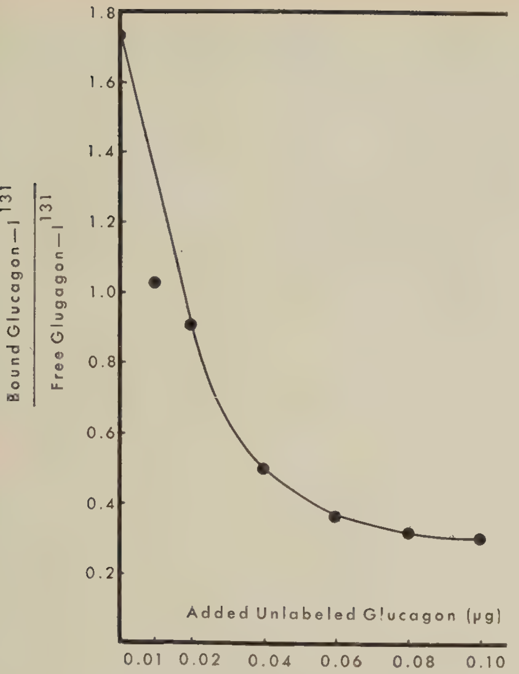

FIG. 2. Addition of increasing amounts of unlabeled glucagon to a mixture of dilute (1:40) antiglucagon serum and glucagon-$I^{131}$ causes a progressive decrease in the ratio of bound to free glucagon-$I^{131}$. The above curve could serve as standard for an immunoassay for glucagon within a range of 0-0.06 µg/cc.

dissociates very rapidly at any time up to 2 hours after its formation.

*Basis of immunoassay for glucagon.* By preincubating unlabeled glucagon in dilute antiglucagon serum it has been possible to reduce the percentage of glucagon-$I^{131}$ bound to globulin. In Fig. 2 the quantity of unlabeled glucagon added, is plotted against the ratio of bound to free glucagon-$I^{131}$ displaced from antibody. It is clear that within a range of 0.01 to 0.06 µg increasing amounts of non-radioactive glucagon displace proportionately increasing amounts of radioactive glucagon from antibody. This would indicate that standard curves for various concentrations of beef-pork glucagon can be constructed and that an immunoassay, at least for beef-pork glucagon, is possible.

*Discussion.* Our data reveal for the first time that specific non-precipitating antibodies to beef-pork glucagon are readily produced in the rabbit. The antigenicity of glucagon was somewhat unexpected in view of its low molecular weight. Furthermore, there was reason to believe that the amino acid sequences of beef and pork glucagon might be identical (personal communication), suggesting that identity in other species might also exist.

The practical implication of this work lies in the demonstration that non-radioactive glucagon competes with radioactive glucagon for glucagon antibody in proportion to its concentration, thereby establishing the basis for a relatively simple immunoassay for glucagon.

*Summary.* Non-precipitating antibodies to glucagon have been produced in rabbits and detected by both Berson-Yalow and Skom-Talmadge technics. A basis for an immunoassay for glucagon appears, thereby, to have been established.

The authors express gratitude to Drs. Solomon A. Berson, Roslyn Yalow, and Marcus Rothschild, N.Y.C.

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# Isoantigenicity of Rabbit Semen.* (25339)

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Isoantigenicity of spermatozoa and testis has been established for a variety of animals (1-4), and autoantibodies to spermatozoa have been found in man in a number of cases (4-9). In view of the close immunological relationship between spermatozoa and seminal plasma, which has been recorded for man(10), guinea pig(11), rabbit(12), and bull(13), we became interested in the isoantigenicity of seminal plasma and its relationship to the antigenic components common to seminal plasma and spermatozoa.

*Methods.* Semen was obtained from mature and fertile, pure bred Dutch belted rabbits (Rockland Farms) by means of the artificial vagina(14,15). Rabbits of mixed breed were used for immunization. After an attempt to elicit antibody response by intravenous injections had failed, antigen was administered deep into the subscapular region in form of a suspension in Freund's adjuvant (Difco). Into 1 ml adjuvant, 1 ml ¼ diluted seminal plasma or 1 x $10^8$ thrice washed spermatozoa in 1 ml saline solution were incorporated. Six injections at 14 day intervals yielded maximal response. Rabbits that showed antibodies in test bleedings were exsanguinated, and the serum stored in dry ice. Immunological methods and preparation of organ extracts used during these experiments have been described(10,12). Absorption of antibody was obtained by adding 500 million washed spermatozoa to 1 ml immune serum. The mixture was incubated at 37°C with frequent shaking, left in the refrigerator over night, and the spermatozoa removed by centrifugation at 10,000 rpm. Organs of immunized and non-immunized (control) rabbits were fixed in formalin and examined after staining with hematoxylin and eosin.†

*Results.* Of 10 rabbits injected with seminal plasma in Freund's adjuvant, 5 (2 male and 3 female) produced antibody demonstrable by complement fixation test, hemagglutination, spermagglutination, and agar diffusion (Ouchterlony's technic). The homologous antisera reacted in complement fixation test with saline extracts of rabbit prostate, but did not react with saline extracts of rabbit testis, epididymis, seminal vesicle, kidney, liver, spleen, muscle, nor with rabbit or human serum, human seminal plasma and spermatozoa (Table I).

Isologous immune serum shows only one precipitation band (Fig. 1). It gave a reaction of identity with the strongest line obtained with heterologous immune serum and could be removed by absorption of the immune serum with either seminal plasma or washed spermatozoa. The cross reactivity of the isologous immune sera against seminal plasma with spermatozoa could also be shown by their ability to agglutinate spermatozoa in high titers (1/1024 or higher).

The complement fixation method did not furnish evidence concerning this point because normal rabbit serum fixes complement in the presence of rabbit spermatozoa in serum dilutions individually varying between 1/10 and 1/160. This was seen with every specimen of 30 rabbits tested, including preimmuniza-

TABLE I. Fixation of Complement by Anti-Rabbit Seminal Plasma Rabbit Immune Serum (Diluted 1/10) and Antigens (Rabbit).

| | A | B | C | D |
|---|---|---|---|---|
| Antigen dilution | Seminal plasma 1/500 | Prostate 1/10 | Testis* 1/10 | Serum 1/100 |
| 1/1 | 4+ | 4+ | — | — |
| 1/2 | 4+ | 4+ | — | — |
| 1/4 | 4+ | 4+ | — | — |
| 1/8 | 4+ | 4+ | — | — |
| 1/16 | 2+ | — | — | — |
| 1/32 | — | — | — | — |

* Negative results were similarly obtained with extracts of epididymis, seminal vesicle, spleen, liver, kidney, and muscle; also with human seminal plasma, spermatozoa and serum. Antigen and antiserum alone, also antigen plus normal rabbit serum remained negative.

4+, no hemolysis; 2+, moderate (approx. 50%) hemolysis; —, complete hemolysis.

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* Supported by grant from N.I.H., U.S.P.H.S.

† We are grateful to Dr. L. Reiner of this Hospital for histological preparations and for confirming results of microscopic examination.

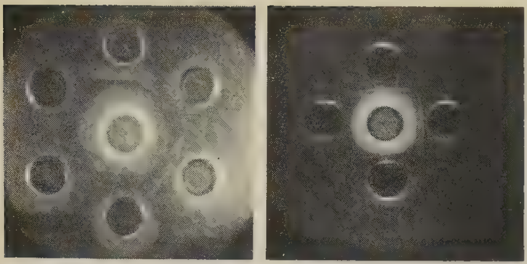

FIG. 1. Center troughs: Rabbit seminal plasma (undiluted). Outer troughs: Sera, undiluted. *Left Frame,* clockwise from top: guinea pig treated with rabbit seminal plasma (4 bands); rabbit treated with rabbit seminal plasma (not reactive); same, reactive (one band); rabbit treated with rabbit spermatozoa (not reactive); same; normal rabbit serum. *Right Frame:* guinea pig treated with rabbit seminal plasma (3 bands); rabbit treated with rabbit seminal plasma (1 band); the same sera absorbed with spermatozoa (no reactions).

tion bleedings from the rabbits subsequently injected with seminal plasma or spermatozoa and the sera of the Dutch belted rabbits serving as donors of the spermatozoa. No consistent increase in titer in terms of effective serum dilution or of concentration of spermatozoa could be observed in our serum specimens containing antibody according to all other criteria. The non-specifically reactive material was liberated from the spermatozoa by sonic vibration(see 10,12). The fluid in which the spermatozoa had been suspended during such treatment showed non-specific reactivity.

Eleven rabbits (5 male and 6 female) were injected with washed rabbit spermatozoa in Freund's adjuvant. No reactions significantly differing from those of pre-immune sera could be obtained in complement fixation tests with spermatozoa as antigen. Thus deprived of our most sensitive tool in testing for circulating antibody, we had to rely on other tests; they remained negative. They include agglutination with seminal and epididymal spermatozoa, hemagglutination, agar diffusion tests and complement fixation test with seminal plasma.

Histological examination of all the rabbits discussed above, including testes and epididymides, showed no degenerative or inflammatory processes.

*Discussion.* Rabbit seminal plasma is isoantigenic. The antibodies produced by rabbits injected with rabbit seminal plasma in Freund's adjuvant are of high specificity. Only one antigenic component could be detected with these antisera by the gel diffusion method. Thus it would appear that only one of the several (at least 5) antigenic components (12) demonstrable with heterologous immune sera is isoantigenic. The antigen involved is present also on spermatozoa obtained from semen. This could be proven by the ability of such spermatozoa to absorb the antibody from iso-immune sera and sperm agglutination.

However, no antibody formation was obtained in rabbits after 6 injections of $10^8$ spermatozoa in Freund's adjuvant or after an extended course of intravenous injections. Why an antigen, which is highly effective in heterologous immunization with spermatozoa, should be ineffective isologically, is not clear. It may be worthy of exploration, because the same antigen is isologically effective in solute form, namely in the seminal plasma, under comparable experimental conditions.

Normal rabbit serum gives complement fixation with rabbit spermatozoa. The nature of this reaction needs to be clarified. It is in sharp contradistinction to the behavior of rabbit serum with the spermatozoa of other species(10) and to that of rabbit spermatozoa with the sera of guinea pig, mouse and man (12).

No degenerative changes are seen in the testes and epididymides of rabbits treated with spermatozoa or seminal plasma. We are grateful to Drs. Bishop and Katsh(16) for their permission to state that they also were unable to obtain degenerative changes in the testes of rabbits injected with testis emulsions in Freund's adjuvant. It would, therefore, appear that the rabbit does not produce the peculiar degenerative changes in the testis and epididymis which characterize the guinea pig's(3,16,17) and (to a lesser degree) the rat's(18) response to spermatozoa or testes suspensions.

*Summary.* 1) Rabbit seminal plasma contains at least one isoantigenically active component. 2) No isoantigenicity could be observed with rabbit spermatozoa under comparable experimental conditions even though the spermatozoa remove homologous antibody against seminal plasma and are agglutinated

by isologous anti-seminal plasma immune serum. 3) Normal rabbit sera fix complement with rabbit spermatozoa. 4) No degenerative damage to rabbit testis or epididymis could be produced by injections of spermatozoa or seminal plasma in Freund's adjuvant.

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# Biological Effects of Hypoglycin A in Hydrocortisone Treated Adrenalectomized Rats.* (25340)

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Hassall and Reyle(1,2) succeeded in isolating 2 peptides, hypoglycin A and B, from fruit seeds of the tree, *Blighia sapida*, and reported them to cause hypoglycemia in rodents. Subsequent structural analyses revealed hypoglycin A to be an amino acid: cyclopropanepropionic acid, α-amino-2-methylene(3,4,5,6). Several authors reported on pharmacological and biochemical actions of hypoglycin A(7,8, 9,10). Attention in this report is directed to action(s) of hypoglycin A on several parameters of hydrocortisone-induced gluconeogenesis.

*Methods.* Adult male Sherman rats (140-170 g) were bilaterally adrenalectomized and maintained with 0.9% sodium chloride solution, along with high protein diet *ad libitum*. On fourth post-operative day the food was removed. Sixteen hours later the rats were injected subcutaneously with 1 mg of hydrocortisone suspended in 0.2 ml of a carboxymethylcellulose vehicle. Hypoglycin A was dissolved in water and 0.2 ml of the solution was injected intraperitoneally at time of steroid administration. Control animals received injections of the vehicles. No food or water was allowed during test period. Thirty minutes after initiating treatment, rats were injected intraperitoneally with 5 ml of 0.9% sodium chloride, and 2 rats placed in each metabolism cage. Four hours after steroid or drug administration animals were anesthetized with sodium pentobarbital and livers were excised and hydrolyzed immediately in 30% potassium hydroxide. During removal of liver, blood was drawn from the portal vein and analyzed for glucose by the Nelson method(11). Liver glycogen was determined by the anthrone method of Seifter, *et al.*(12).

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* This work was performed in the Pharmacological Research Dept., Dr. Earl H. Dearborn, Head.

TABLE I. Effects of Hypoglycin A on Liver Glycogen, Blood Glucose and Urinary Glucose and Nitrogen in Fasted Adrenalectomized Rats Treated with Hydrocortisone: 4 Hour Assay.

| Dose, mg | | | | | | |
|---|---|---|---|---|---|---|
| Hydrocortisone | Hypoglycin A | No. rats | Liver glycogen, mg/g liver | Blood glucose, mg % | Urine glucose,* mg % | Total urinary nitrogen, mg |
| | | 4 | 2.6 (2.1– 3.3)† | 76 ( 64– 84) | 73 (68– 78) | 21 (14–29) |
| 1.0 | | 4 | 8.1‡ (7.0–11.3) | 98‡ ( 88–106) | 39 (38– 40) | 48 (38–58) |
| 1.0 | .125 | 4 | 8.1 (7.8– 8.9) | 106‡ ( 98–116) | 57 (50– 64) | 51 (19–82) |
| 1.0 | .25 | 4 | 6.2 (3.1– 8.1) | 108‡ (102–116) | 59 (54– 64) | 27 (17–37) |
| 1.0 | .50 | 4 | 6.0 (4.7– 7.7) | 106‡ (102–112) | 62 (50– 74) | 39 (30–48) |
| 1.0 | 1.0 | 4 | 5.1§ (4.5– 5.6) | 105‡ ( 92–116) | 48 (44– 52) | 45 (35–55) |
| 1.0 | 2.0 | 4 | 5.4§ (4.7– 6.3) | 106‡ (102–112) | 56 (50– 62) | 49 (44–54) |
| 1.0 | 4.0 | 4 | 10.8 (9.0–14.1) | 103‡ ( 98–106) | 61 (52– 70) | 47 (42–51) |
| 1.0 | 8.0 | 4 | 2.9§ (1.8– 4.4) | 93‡ ( 84– 98) | 91 (62–120) | 56 (42–70) |
| 1.0 | 16.0 | 4 | 3.2§ (2.6– 3.9) | 72 ( 60– 82) | 70 | 26 |

* Total reducing substances as measured by Nelson method(11).

† Mean (range).

‡ $P < .05$ when compared to untreated controls.

§ $P < .05$ " " " hydrocortisone-treated controls.

No gross hypoglycemic symptoms observed.

Glucose was assayed in aliquots of urine by the Nelson method(11), and nitrogen was analyzed by the micromethod of Shepherd, *et al.*(13). Liver glycogen deposition and blood glucose data were statistically analyzed by unpaired replicate ranking procedure of Wilcoxon(14). Analysis of urinary glucose and nitrogen excretion figures was prohibited since there were only 2 observations/group for each parameter.

*Results.* Rats injected with hydrocortisone alone exhibited a significant increase in liver glycogen ($P<0.05$), blood glucose ($P<0.05$) and urinary nitrogen excretion as compared to untreated controls (Table I). There also was a suggested decrease in urinary glucose levels. Hydrocortisone-stimulated liver glycogen deposition was reduced in animals receiving 1, 2, 8 and 16 mg of hypoglycin A. The aberrant glycogen value recorded for the group injected with 4 mg was unexplainable. Blood glucose concentrations were reduced in rats treated with 16 mg of hypoglycin A. These data suggest that depletion of liver glycogen precedes reduction of blood glucose. Urinary glucose values were apparently unaffected by hypoglycin A. Urinary nitrogen excretion also appeared unaltered.

*Discussion.* Feng and Patrick(8) reported that the most outstanding change produced by hypoglycin A was a delayed hypoglycemia, preceded by exhaustion of liver glycogen. Hypoglycemic activity also has been demonstrated in adrenalectomized animals(7,9). The present evidence indicates that hypoglycin A is capable of antagonizing the action of hydrocortisone as evidenced by decreases in both liver glycogen deposition and hyperglycemia. It is suggested that this antagonism is not mediated through: 1) a "phlorhizin-like action" since no increase in urinary glucose was observed, or 2) interference with conversion of protein to carbohydrate intermediates inasmuch as urinary nitrogen excretion was, in general, unaltered. These results are consistent with the hypothesis(8) that the primary action of hypoglycin A is the interference with hepatic glycogen production.

*Summary.* Administration of hypoglycin A (cyclopropanepropionic acid, α-amino-2-methylene) to adrenalectomized male rats reduced the degree of hydrocortisone-induced liver glycogen deposition and hyperglycemia, without altering either urinary glucose or nitrogen excretion.

The authors express their thanks to Dr. R. S. DeRopp and associates of the Biochemistry Dept. for supplies of hypoglycin A, and also to W. Cekleniak for aid in conducting glucose and nitrogen analyses.

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## Adrenal Corticoid Activities of $\Delta^1$, 9$\alpha$ fluoro-hydrocortisone 16$\alpha$, 17$\alpha$ isopropylidenedioxy.*† (25341)

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Important chemical transformations which augmented the mineralo- and/or glucocorticoid actions of hydrocortisone include: halogenation at C-6(1), C-9(2) and C-12(3); introduction of a double bond between C-1 and C-2(4); and methylation at C-2(5), C-6 (6) and C-16(7). Bernstein and associates (8-12) dissociated the undesirable sodium-retentive activity of certain potent glucocorticoids by inclusion of 16$\alpha$ hydroxyl group. Fried, *et al.*(13) recently reported that 16$\alpha$, 17$\alpha$ diol groups of $\Delta^1$, 16$\alpha$ hydroxy, 9$\alpha$ fluorohydrocortisone reacted with various aldehydes and ketones to form cyclic acetals and ketals, which exhibit greater glucocorticoid activity than the parent steroid. This report details the biological activities of $\Delta^1$, 9$\alpha$ fluoro-hydrocortisone, 16$\alpha$, 17$\alpha$ isopropylidenedioxy (triamcinolone acetonide).

*Materials and methods.* A 3-day bioassay was employed in which both liver glycogen deposition and thymus involution were determined in each of a group of adrenalectomized immature male rats. Urine volume and electrolyte excretion also were measured. Immature male rats of Sherman strain, weighing 40-60 g, were bilaterally adrenalectomized. Animals were maintained with Purina Lab. Chow and 1% NaCl drinking fluid *ad lib.* Twenty-four hours after adrenalectomy the rats were injected subcutaneously or given by gavage graded doses of steroid suspended in 0.2 ml of a modified carboxymethylcellulose vehicle (14). Control animals were injected with vehicle only. One-half hour after administration of the steroid the rats were injected intraperitoneally with 3 ml of 0.9% saline and 3 rats placed in each metabolism cage without food or water. Five hours later urine volume was measured and the cages rinsed with distilled water. The animals were then removed from the metabolism cage and returned to holding racks containing the stock diet and saline drinking fluid. Total milliequivalents of sodium and potassium in urine samples were determined by flame photometry. Steroid was administered daily for the next 2 days. The

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* The generic name of this steroid is triamcinolone acetonide. It is currently being marketed as a topical anti-inflammatory agent under trademarks Aristocort® Triamcinolone Acetonide Cream (Am. Cyanamid Co.) and Kenalog® (E. R. Squibb & Sons).

† The authors are indebted to Dr. S. Bernstein and associates for generous supplies of steroids, and J. D. Haynes for statistical analyses.

rats were fasted 15 hours prior to and 7 hours after the last injection to insure maximum depletion of liver glycogen. Animals were then anesthetized with sodium pentobarbital and the livers quickly excised and hydrolyzed in 30% potassium hydroxide(15). Thymi were removed and weighed on Roller-Smith Torsion balance. Liver glycogen was measured by the anthrone method of Seifter, *et al.*(16). Data were statistically analyzed by fitting straight lines by the method of least squares and computing the corresponding equation. Life-maintenance and growth of adrenalectomized, immature male rats were studied by a method previously described(4). However, since analyses of data indicated that survival time of adrenalectomized rats treated with corticosteroids did not follow a normal distribution, results were plotted on *log*-probability paper and expressed as median survival time.

*Results.* Triamcinolone acetonide and triamcinolone were each compared with hydrocortisone for liver glycogen deposition and thymolytic activities by both subcutaneous and oral routes (Table I). Triamcinolone acetonide was 21 times as potent as hydrocortisone subcutaneously and 167 times hydrocortisone orally (Table II). Subcutaneously administered triamcinolone was 6 times more potent than hydrocortisone and 42 times hydrocortisone orally. These values for triamcinolone are somewhat lower than previously reported(14); however, this apparent disparity is presumably due to differences in assay procedures.

Table I depicts comparative activities of triamcinolone acetonide and triamcinolone on thymus involution. Triamcinolone acetonide was 29 times more potent than hydrocortisone subcutaneously, and 79 times hydrocortisone orally (Table II). Relative potencies of triamcinolone to hydrocortisone on subcutaneous and oral assays were calculated to be 4 and 5, respectively.

Triamcinolone acetonide and triamcinolone exhibited marked ability to promote sodium, potassium and water excretion when administered subcutaneously (Fig. 1). Similar results were obtained when the steroid was given orally. Potassium and water excretion appeared to have increased over control levels to a greater degree than did sodium excretion.

TABLE I. Oral and Subcutaneous Liver Glycogen Deposition and Thymus Involution Activity of Hydrocortisone, Triamcinolone and Triamcinolone Acetonide.

| Compound | Dose ($\mu$g/rat) | Liver glycogen* (mg/g liver) | Thymus wt* (mg/100 g body wt) |
|---|---|---|---|
| | | Subcut. | |
| Control | | 2.03 ± .12† | 407 ± 18† |
| Hydrocortisone | 200 | 7.37 ± .82 | 302 ± 21 |
| | 400 | 12.82 ± .88 | 215 ± 16 |
| | 700 | 26.83 ± 2.78 | 124 ± 7 |
| | 1000 | 54.60 ± 4.23 | 83 ± 5 |
| | 1300 | 57.80 ± 2.46 | 75 ± 8 |
| Triamcinolone | 25 | 6.52 ± .81 | 341 ± 15 |
| | 50 | 11.80 ± 1.05 | 284 ± 11 |
| | 100 | 35.07 ± 5.63 | 184 ± 13 |
| | 200 | 68.12 ± 3.04 | 94 ± 4 |
| | 400 | 77.60 ± 4.19 | 80 ± 3 |
| Triamcinolone acetonide | 6.25 | 8.41 ± 3.20 | 221 ± 22 |
| | 12.5 | 10.20 ± 1.20 | 179 ± 13 |
| | 25 | 16.48 ± 2.22 | 131 ± 9 |
| | 50 | 45.42 ± 1.85 | 79 ± 5 |
| | 100 | 69.51 ± 5.25 | 68 ± 5 |
| | | Oral | |
| Control | | 2.10 ± .20 | 390 ± 21 |
| Hydrocortisone | 500 | 4.24 ± 1.11 | 274 ± 18 |
| | 1000 | 6.52 ± .86 | 211 ± 21 |
| | 2000 | 16.06 ± 1.86 | 137 ± 15 |
| | 4000 | 21.77 ± 6.97 | 123 ± 8 |
| | 8000 | 28.51 ± 4.22 | 88 ± 4 |
| Triamcinolone | 100 | 15.18 ± 1.87 | 296 ± 20 |
| | 200 | 26.03 ± 3.75 | 226 ± 23 |
| | 400 | 40.27 ± 4.43 | 146 ± 16 |
| | 800 | | 110 ± 7 |
| Triamcinolone acetonide | 12.5 | 15.39 ± 1.82 | 207 ± 20 |
| | 25 | 24.90 ± 3.72 | 156 ± 15 |
| | 50 | 29.36 ± 3.72 | 106 ± 6 |
| | 100 | 36.72 ± 5.96 | 80 ± 3 |
| | 200 | 52.17 ± 5.28 | 68 ± 6 |

* 10-18 rats/dose. † Mean ± stand. error.

A graphic representation of life-maintenance data is shown in Fig. 2. Doses of steroids resulting in median survival time of 10 days were as follows: desoxycorticosterone, 187 $\mu$g (95% confidence limits 129 and 271); triamcinolone, 19.9 $\mu$g (14.5 and 27.3); and triamcinolone acetonide, 1.4 $\mu$g (1.0 and 1.9). The potencies of triamcinolone and triamcinolone acetonide relative to desoxycorticosterone were 9 and 132, respectively (Table II).

*Discussion.* The reaction of triamcinolone and acetone to form the cyclic derivative, triamcinolone acetonide, results in a steroid possessing greater glucocorticoid activity than

TABLE II. Comparative Adrenocorticoid Activities.

| | | Relative potencies | | |
|---|---|---|---|---|
| Steroid | Route of admin. | Glycogen deposition (× hydrocortisone) | Thymus involution (× hydrocortisone) | Life maintenance (× DOC) |
| Hydrocortisone | Subcut. | 1 | 1 | |
| Desoxycorticosterone | | | | 1 |
| Triamcinolone | | 6 (5 & 7)* | 4 (3 & 4) | 9 (5 & 19) |
| " acetonide | | 21 (18 & 24) | 29 (25 & 34) | 132 (71 & 244) |
| Hydrocortisone | Oral | 1 | 1 | |
| Triamcinolone | | 42 (29 & 61) | 5 (3 & 6) | |
| " acetonide | | 167 (114 & 244) | 79 (56 & 112) | |

* 95% confidence limits.

the parent compound. Compared to hydrocortisone by subcutaneous liver glycogen assay method the following activities were determined: triamcinolone, 6 (95% confidence limits 5 and 7) and triamcinolone acetonide, 21 (18 and 24). Thymolytic activities were: triamcinolone 4 (3 and 4) and triamcinolone acetonide 29 (25 and 34). Oral administration of triamcinolone and the cyclic derivative resulted in a further enhancement of the parenteral liver glycogen deposition activity. Table II shows that there was no increase in thymolytic activity of triamcinolone when given orally, while triamcinolone acetonide showed a marked increase. At present, there is no rationale for this observation.

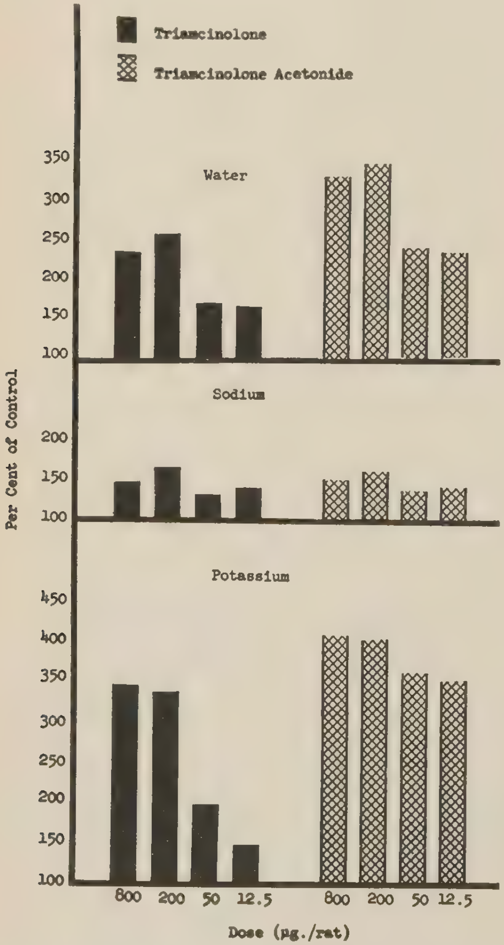

FIG. 1. Diuretic, natriuretic and kaliuretic activities of triamcinolone and triamcinolone acetonide.

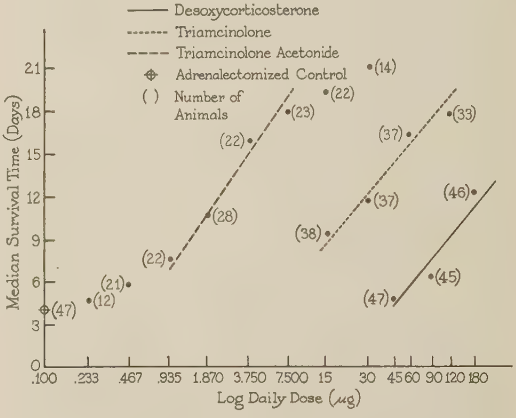

FIG. 2. Life-maintenance activities of desoxycorticosterone, triamcinolone and triamcinolone acetonide.

Though in animal assays, orally administered triamcinolone acetonide is appreciably more active than the parent steroid, clinical investigations have shown that triamcinolone acetonide, given orally to patients with diseases that ordinarily respond to steroid therapy, was equivalent in activity, on a weight basis, to triamcinolone free alcohol (Lederle Labs., personal communication). Nevertheless, dermatological studies have shown that 0.1% triamcinolone acetonide cream was more efficacious than 1% hydrocortisone cream(17). These clinical results further emphasize the disparity which may occur between assessments of corticosteroid activity in human subjects and laboratory animals.

Although the ketal analogue of triamcinolone possesses greater glucocorticoid activity than the parent steroid, effects on water, sodium and potassium diureses are comparable. The diuretic and natriuretic potencies of triamcinolone acetonide have been corroborated using normal intact rats and dogs (Cummings, J. J., personal communication).

Survival of adrenalectomized immature male rats following multiple injections of steroids indicates that triamcinolone is 9 (95% confidence limits 5 and 19) and triamcinolone acetonide is 132 (71 and 244) times more active than desoxycorticosterone. Triamcinolone acetonide is 14 (9 and 23) times more effective than triamcinolone. Desoxycorticosterone proved superior to either triamcinolone or triamcinolone acetonide in promoting body weight gain. These results are similar to those previously reported for desoxycorticosterone and triamcinolone(14).

*Summary.* Glucocorticoid properties of subcutaneously and orally administered triamcinolone acetonide were assayed in immature adrenalectomized rats by liver glycogen deposition and thymus involution. Subcutaneously, triamcinolone acetonide is about 3 times as potent as triamcinolone on liver glycogen assay and 7 times triamcinolone on the thymolytic assay. Oral activities of triamcinolone acetonide also were greater than those of the parent steroid. Triamcinolone acetonide was capable of inducing a diuretic, natriuretic and kaliuretic response in adrenalectomized rats. Triamcinolone acetonide was more active than either desoxycorticosterone or triamcinolone for maintaining the life of immature, adrenalectomized male rats.

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# Enhanced Sensitization in Mice by Simultaneous Injection of Antigen and Specific Rabbit Antiserum.* (25342)

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In following the degradation of $I^{131}$ labeled human serum albumin ($I^{131}$ HSA) in mice passively sensitized with rabbit antiserum, it was noted that a *second* phase of accelerated degradation of $I^{131}$ HSA occurred approximately 100 hours following the $I^{131}$ HSA-antiserum injection. In contrast, control mice injected only with $I^{131}$ HSA degraded the labeled protein at a constant rate. This suggested that as a result of injecting mice intravenously with HSA-rabbit HSA antiserum, a greater antibody response was elicited to HSA than was elicited to HSA alone. The present study demonstrates that in mice, a significantly greater antibody response is elicited to serum albumin (human or bovine) by intravenous injection of serum albumin plus specific rabbit antiserum (in serum albumin excess) than is elicited by intravenous injection of serum albumin alone. The experimental design was as follows: Mice were divided into groups and injected intravenously with (1) serum albumin mixed *in vitro* with specific rabbit antiserum (*experimental*), (2) serum albumin plus normal rabbit serum (*NS controls*), or (3) serum albumin alone (*controls*). To determine if mice had become actively sensitized, they were challenged intravenously with $I^{131}$ labeled serum albumin. Anaphylaxis was graded and the amount of $I^{131}$ labeled serum albumin degraded in the ensuing 24 hours was determined. (In passively sensitized mice, the *amount* of antigen degraded at the acceleratd rate is a function of amount of antibody used(1)).

*Materials and methods. Mice.* Eight-week-old Swiss albino (Hale-Stoner) mice were used. $I^{131}$ labeled human serum albumin ($I^{131}$ HSA) was obtained commercially (Abbott Labs). Crystalline bovine serum albumin (BSA) was purchased from Armour Labs and iodinated ($I^{131}$) using $KI_3$(2). Rabbits were injected intravenously with 10 mg of serum albumin (HSA or BSA) 3 times a week for 6 weeks. One week following last injection, the rabbits were bled and sera pooled. The rabbit anti-HSA serum contained 3.2 mg of anti-HSA antibody/ml, and rabbit anti-BSA serum contained 2 mg of anti-BSA antibody/ml. *Gradation of anaphylaxis.* In mice *mild* shock is characterized by hyperexcitability to stimulation, absence of spontaneous movement, a hunched posture, dilation of blood vessels in the ear and labored breathing. *Moderate* shock is characterized by hyperexcitability, sluggishness of movement, difficulty with breathing and cyanosis of ears and feet. The hunched posture is absent. *Severe* shock is characterized by complete immobility, shallow breathing and intermittent convulsions. The degradation of $I^{131}$ HSA and $I^{131}$ BSA was followed by whole-body measurement of radioactivity(1). *Exp. I, HSA*: *Experimental group.* Eleven mice were injected intravenously with $I^{131}$ HSA plus rabbit anti-HSA serum (0.15 ml). Rabbit anti-HSA serum diluted 1 to 4 with saline (1% NaCl) was mixed *in vitro* with equal volume of $I^{131}$ HSA solution (0.5 mg/ml), and stored at 37°C for 2 hours. No antigen-antibody precipitate was detected in the $I^{131}$ HSA-antiserum solution. The amount of $I^{131}$ HSA added to antiserum was 2.5 to 3 times the amount necessary to produce maximum precipitation. *NS control group.* Eight mice were injected with a solution of normal rabbit serum plus $I^{131}$ HSA (0.2 ml). Normal rabbit serum was diluted 1 to 4 with saline, mixed with equal volume of $I^{131}$ HSA (0.5 mg/ml), and stored at 37°C for 2 hours prior to injection. *Control group.* Nine mice were injected intravenously with 0.025 mg of $I^{131}$ HSA. After onset of second phase of accelerated degradation of $I^{131}$ HSA in mice of the experimental group, all mice of the 3 groups were challenged (6th day) intravenously with 0.01 mg of $I^{131}$ HSA. Anaphylaxis was graded and degradation of $I^{131}$ HSA was followed. The *NS control* mice were

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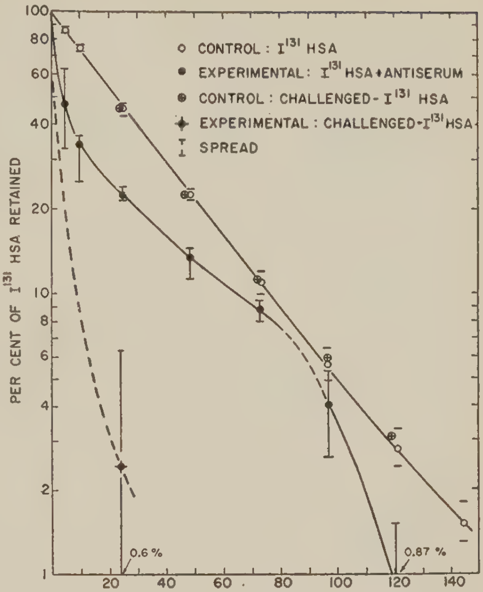
* Research supported by U. S. Atomic Energy Comm.

again challenged on 10th day with $I^{131}$ HSA, anaphylaxis was graded, and degradation of $I^{131}$ HSA was followed. *Exp. II, BSA*: *Experimental group*. Seventeen mice were injected intravenously with BSA plus rabbit anti-BSA serum (0.2 ml). Rabbit anti-BSA serum diluted 1 to 2 with 1% saline was mixed with an equal volume of BSA (1 mg BSA/ml). The amount of BSA used was approximately 3 times the amount necessary to produce maximum precipitation. The BSA-rabbit anti-BSA serum solution was injected immediately after mixing. *Control Group I*. Sixteen mice were injected intravenously with 0.1 mg of BSA alone. On 7th day, all mice were challenged intravenously with 0.15 mg of $I^{131}$ BSA. Eight mice not previously exposed to BSA were also injected intravenously with 0.15 mg of $I^{131}$ BSA (Control Group II). Anaphylaxis was graded and amount of $I^{131}$ BSA degraded in the ensuing 24 hours was determined.

*Results*. *Exp. I*: The results obtained with *experimental* and *control* mice are presented in Fig. 1. The range in values at each measurement is designated by bars. In *experimental* mice (solid circle), the $I^{131}$ HSA was degraded initially at an accelerated rate and later at a slower rate. Between 70 and 96 hours following administration of HSA-anti-HSA, a break in the retention curve occurred due to accelerated degradation of $I^{131}$ HSA (2nd phase). With *control* mice a straight line was obtained throughout. On 6th day, the mice were challenged. The results are summarized in Table I. All *experimental* mice developed mild or moderate anaphylactic shock, while none of the *control* mice was anaphylactically shocked. *Experimental* mice (solid circle with a cross; Fig. 1) degraded 94 to 99% of the $I^{131}$ HSA in the 24-hour period following challenge; control mice degraded 55% of the $I^{131}$ HSA in the same period. The degradation of $I^{131}$ HSA in the *control* mice was followed an additional 100 hours and the results duplicated those following the first $I^{131}$ HSA injection.

The results obtained with *NS control* mice are presented in Fig. 2. None of these mice was anaphylactically shocked at either challenge (6th and 10th days). Following the 2 challenges, $I^{131}$ HSA was degraded at the same rate as that observed following initial $I^{131}$ HSA injection. The degradation of $I^{131}$ HSA was followed a total of 13 days.



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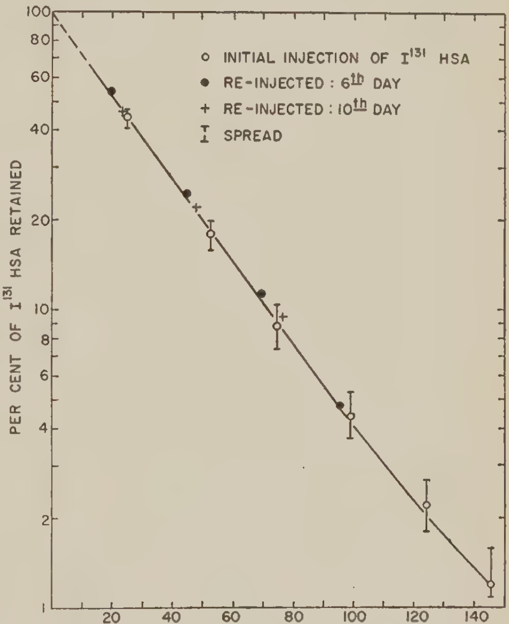

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FIG. 1. The degradation of $I^{131}$ HSA in control mice contrasted with its degradation when given with rabbit anti-HSA.

FIG. 2. The degradation of $I^{131}$ HSA in mice inj. with $I^{131}$ HSA and normal rabbit serum.

TABLE I. Summary of the Results of Exp. I.

| | | | Challenge* (6th day) | |
|---|---|---|---|---|
| Group | No. of mice | Initial inj. | Anaphylactic shock | % of $I^{131}$ HSA retained at 24 hr |
| Experimental | 11 | $I^{131}$ HSA + rabbit antiserum | Mild to moderate | .6 to 6.0 |
| NRS control | 8 | $I^{131}$ HSA + NRS | None | 52.0 to 57.0† |
| Control | 9 | $I^{131}$ HSA | " | 40.0 to 48.0 |

* Intravenous: 0.01 mg of $I^{131}$ HSA.

† Determined at 19 hr.

*Exp. II*: The results using BSA with or without rabbit anti-BSA serum are summarized in Table II. All *experimental* mice were anaphylactically shocked and the amount of $I^{131}$ BSA degraded in the 24-hour period following the challenge ranged from 82 to 93% of amount injected. None of the *control* mice was anaphylactically shocked and these mice degraded approximately the same amount of $I^{131}$ BSA as did control mice on the challenge (control group II).

*Discussion.* These results lead to the conclusion that a significantly greater antibody response to serum albumin (human or bovine) is elicited when mice are injected intravenously with a solution of serum albumin and specific rabbit antiserum in antigen excess, than is elicited in mice injected wth serum albumin alone, or with serum albumin and normal rabbit serum. Thus the *experimental mice* given HSA and rabbit anti-HSA serum when challenged intravenously on the 6th day were anaphylactically shocked, and in the ensuing 24 hours degraded 94% or more of the $I^{131}$ HSA used in the challenge. In contrast, none of the control mice was anaphylactically shocked and all control mice degraded $I^{131}$ HSA at a rate identical to that following initial $I^{131}$ HSA injection, indicating that none of these mice became actively sensitized to HSA. Similar results were obtained with the BSA system (Exp. II).

It is impossible to state unequivocally that control mice did not respond immunologically to HSA or BSA. A positive response might have been obtained with control mice, had a more sensitive method been employed to measure antibody response. With the method employed (antigen degradation), greater sensitivity could have been obtained had a smaller quantity of $I^{131}$ labeled protein been used in the challenge, since the *per cent* of antigen degraded at an accelerated rate is a function of the amount of antigen used(1).

TABLE II. Summary of the Results of Exp. II.

| | | | | Challenge* (7th day) | |
|---|---|---|---|---|---|
| Group | No. of mice | Initial inj. | Mouse | Anaphylactic shock | % of $I^{131}$ BSA retained at 24 hr |
| Experimental | 17 | BSA + rabbit antiserum | 1 | Fatal | |
| | | | 2 | Moderate | 3 |
| | | | 3 | " | 3 |
| | | | 4 | " | 4 |
| | | | 5 | " | 5 |
| | | | 6 | " | 5 |
| | | | 7 | " | 7 |
| | | | 8 | " | 7 |
| | | | 9 | " | 7 |
| | | | 10 | " | 13 |
| | | | 11 | Mild | 4 |
| | | | 12 | " | 11 |
| | | | 13 | " | 12 |
| | | | 14 | " | 13 |
| | | | 15 | " | 14 |
| | | | 16 | " | 15 |
| | | | 17 | " | 18 |
| Control I | 16 | BSA | | None | 29.10 ± .04 |
| " † II | 8 | | | " | 26.50 ± .30 |

* Intravenous: 0.15 mg of $I^{131}$ BSA.

† Control mice on the challenge.

Mice can be actively sensitized to either HSA or BSA with multiple injections. Three interperitoneal injections of HSA (10 mg/injection, 7 days between injections) resulted in 50% of mice being anaphylactically shocked when challenged intravenously 21 days following the first injection(3). Mice were not anaphylactically shocked when challenged 7 or 21 days following a single intraperitoneal injection of HSA (30 mg)(3). Four subcutaneous injections of BSA (4 mg/injection) administered on an every-other-day schedule resulted in approximately 75% of mice being anaphylactically shocked when challenged 14 days following first injection(4). A single subcutaneous injection of BSA (16 mg) results in only 1 or 2% being anaphylactically shocked when challenged on 14th day. Thus in comparison, a single intravenous injection of HSA or BSA (about 0.05 mg) plus its specific antiserum is as effective in actively sensitizing mice to serum albumin as multiple (interperitoneal or subcutaneous) injection of serum albumin alone.

Several studies have been reported in which antigen and antibody were combined and active sensitization measured (*e.g.*, 5,6,7). None of these, of which we are aware, revealed the marked differences in response here described (HSA alone *vs.* HSA plus antiserum). In fact, the opposite effect has been reported(7). Thus ovalbumin-rabbit antiovalbumin precipitates were incorporated in adjuvant and injected into foot pads of guinea pigs. These precipitates were formed in both antigen and antibody excess. Other guinea pigs were similarly injected with an equal amount of "free" ovalbumin. When these animals were challenged with ovalbumin 2 to 3 weeks later, it was found that a higher degree of sensitivity had been achieved with "free" ovalbumin than with the immune precipitates.

*Summary.* Mice were injected intravenously with a solution of serum albumin (human and bovine) plus specific rabbit antiserum in antigen excess. The antibody response to serum albumin was tested and compared to that obtained in mice injected intravenously with serum albumin alone or with albumin and normal rabbit serum. The results show that antibody response elicited with serum albumin plus specific rabbit antiserum is significantly greater than that elicited using serum albumin alone or with normal rabbit serum.

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# Level of 3-Methylcholanthrene in Mammary Glands of Rats after Intragastric Instillation of Carcinogen.* (25343)

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(Introduced by Julian L. Ambrus)
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Development of mammary adenocarcinoma in rats following oral administration of 3-methylcholanthrene has been reported(1,2). These observations also indicated that induced mammary cancers were hormone-dependent. Our studies have further demonstrated that, in rats, steroid hormones promote mammary carcinogenesis by 3-methylcholanthrene(3). In earlier reports by Shay *et al.*(1) and later by Huggins *et al.*(2) and by Dao *et al.*(3),

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* This study was supported in part by grants from Nat. Cancer Inst., U.S.P.H.S.

TABLE I. Levels of 3-Methylcholanthrene in Tissues of 50-Day-Old Virgin Female Rats Fed the Hydrocarbon.

| Tissue | Level of 3-methylcholanthrene (µg/g tissue) |
|---|---|
| Breast, rat 1 | 15 |
| Fat, ” | 70 |
| Breast, rat 2 | 12 |
| Fat, ” | 17 |
| Breast, rat 3 | 24 |
| Fat, ” | 38 |
| Brain, pooled | 3.5 |
| Lung, ” | 2.1 |
| Kidney, ” | 1.1 |
| Thymus, ” | 1.4 |
| Liver, ” | .3 |
| Muscle, ” | .04 |
| Heart, ” | * |
| Spleen, ” | * |
| Uterus, ” | * |
| Ovaries, ” | * |
| Adrenals, ” | * |
| Pituitaries, ” | * |

* Not found.

Total content of 3-methylcholanthrene was less than 0.1 µg in pooled tissues of 3 rats.

mammary tumors were the only neoplasms observed in rats fed with 3-methylcholanthrene. Selective response of mammary tissue to orally administered 3-methylcholanthrene cannot be explained fully, since the mechanism of carcinogenesis is not known. However, reports(4,5,6,7,8,9,10) have demonstrated selective concentration of administered polycyclic carcinogens in breast and fat, and in milk of lactating females of various animals. The earlier reports, however, were concerned primarily with qualitative demonstration of compounds in tissues because of lack of instruments for measuring concentrations of the polycyclic hydrocarbons in the presence of tissue impurities. With recent development of spectrophotofluorometers, it has been possible to determine distribution of 3-methylcholanthrene in tissues on a quantitative basis and to carry out detailed clearance studies in specific tissues. Because of specific effects of 3-methylcholanthrene upon rat breast, results of preliminary studies are of general interest.

*Method.* 3-methylcholanthrene was fed through a stomach tube, a firm rubber (#10 F) catheter, 5 to 6 cm long, attached to the adapter of a syringe. In the first experiment, a single feeding of 30 mg of 3-methylcholanthrene in 1 ml of sesame oil was given to 3 virgin Sprague-Dawley Albino female rats at 50 days of age and a single feeding of 10 mg/ml sesame oil to 3 lactating rats 7 days after parturition. Twenty-four hours later, animals were sacrificed; tissues as listed in Table I were removed for analysis. In the study in which the content of 3-methylcholanthrene in milk was examined, the milk was obtained from the stomach of offspring of females fed 10 mg of 3-methylcholanthrene 24 hours earlier. In a second experiment, breast and abdominal and retroperitoneal fat were removed from animals that had received one single feeding of 30 mg of 3-methylcholanthrene and had been sacrificed at intervals of 1, 2, 3, 4, and 8 days. All dissecting instruments and glassware were specially cleaned to avoid contamination with methylcholanthrene. Tissues were weighed, minced, and treated with equal volumes (ml/g tissue) of 95% ethanol and 10% aqueous potassium hydroxide. They were heated under reflux for 6 to 8 hours, then extracted twice with benzene. The pooled benzene fractions were examined in the Aminco-Bowman Spectrophotofluorometer using either 295 mµ or 355 mµ excitation light and measuring the 410 mµ emission peak. Crude extracts of breast, fat, milk, brain, lung, and thymus could be analyzed directly (Fig. 1a, 1b). Other tissues contained low ratios of 3-methylcholanthrene to interfering substances and therefore had to be purified further. Purification was achieved by removal of benzene from the extract, which was then taken up in cyclohexane and passed through a silica gel column. The column was washed with cyclohexane, then 3-methylcholanthrene was eluted with benzene. With this procedure, 3-methylcholanthrene levels could be determined in liver and kidney. At time of analysis, each sample was read, diluted, and read again, to ascertain that the photometer response was proportional to concentration. A reference standard was read before and after each sample. Recovery of known amounts of 3-methylcholanthrene from tissues was between 85 and 95%, using the complete procedure.

*Results* show that 24 hours after oral administration of 3-methylcholanthrene to rats,

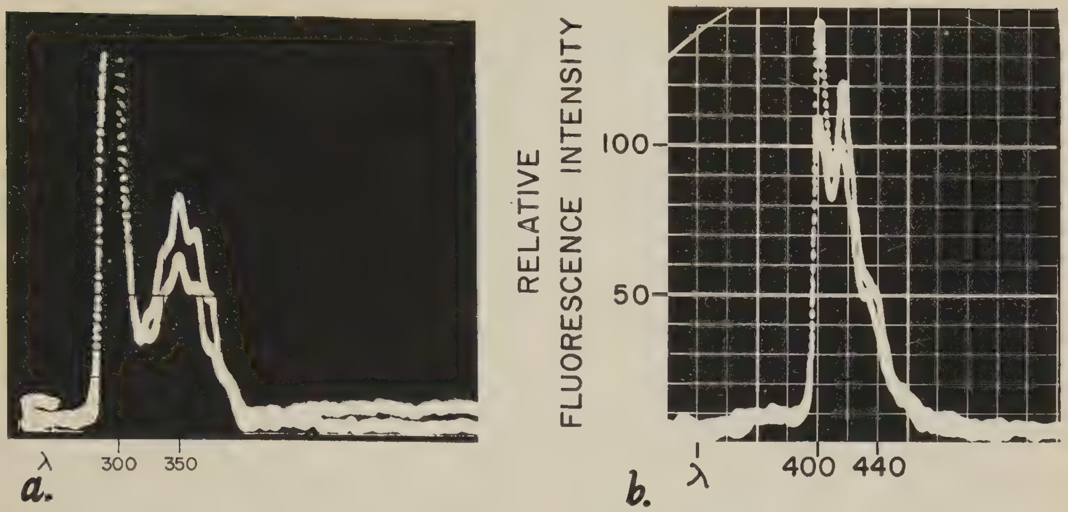

FIG. 1a. Excitation spectrum. Upper curve, 3-methylcholanthrene, 1 $\gamma$/ml; lower curve, crude extract of hydrolyzed milk curd. Emitted light = 410 m$\mu$.

FIG. 1b. Emission spectrum. Upper curve, 3-methylcholanthrene; lower curve, crude extract of hydrolyzed milk curd. Excitation light = 295 m$\mu$.

the aromatic hydrocarbon is found mainly in fatty and breast tissues (Table I) while levels in other tissues are either very small or insignificant. It also seems evident that levels of 3-methylcholanthrene are higher in fat than those in breast tissues. However, in the lactating breast, the level of 3-methylcholanthrene is reversed, higher in breast tissue and lower in fat. Milk curd from fetuses, of the stomach contained high concentration of 3-methylcholanthrene after one single feeding of the carcinogen.

Results of second experiment show that the level of 3-methylcholanthrene decreases rapidly in the breast and fat within 72 hours, but a significant amount of 3-methylcholanthrene remains in both tissues even at end of 8 days (Fig. 2). Rate of clearance of ingested 3-methylcholanthrene is about the same in both fat and breast tissues.

*Discussion.* It is evident from our results that both fat and breast tissues contain high concentrations of 3-methylcholanthrene after a single feeding of the hydrocarbon. Breast tissue from rats contained a large amount of adipose tissue, which may have accounted for much of the 3-methylcholanthrene. However, the high levels of 3-methylcholanthrene in breasts as well as the high content of the carcinogen in milk of lactating rats suggest that 3-methylcholanthrene was present in glandular cells of mammary glands since it is from these cells the milk is produced. Furthermore, the fact that the 3-methylcholanthrene in the lactating breast tissue is higher than that of the fat is indicative of the presence of carcinogen in cells of a hyperfunctioning mammary gland (Table II).

It is believed that carcinogens take a considerable time to manifest their effects. Dur-

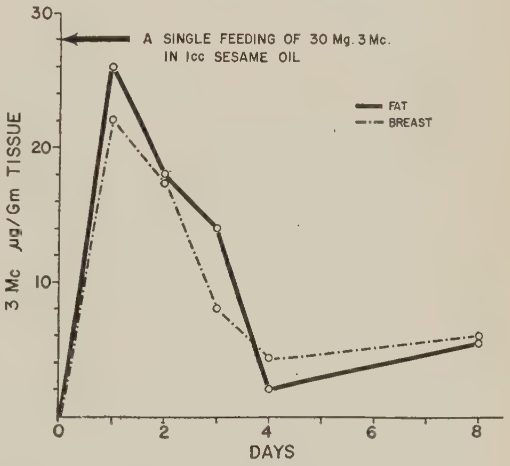

FIG. 2. Clearance of 3-methylcholanthrene from the breast and fat following a single feeding of 30 mg of the carcinogen in 50-day-old female Sprague-Dawley rat. Note persistence of 3-methylcholanthrene in these tissues 8 days following single feeding. 3 Mc = 3-methylcholanthrene.

TABLE II. Levels of 3-Methylcholanthrene in Breast and Fat of Virgin Rats and in Milk, Breast and Fat of Lactating Rats. One single feeding of 3-methylcholanthrene of 10 mg/cc sesame oil was given and animals were sacrificed 24 hr later.

| | | Tissues | Wt (g) | 3-methylcholanthrene ($\mu$g/g tissue) |
|---|---|---|---|---|
| Virgin rats 50 days old | I | Breast | 2.6 | 6.5 |
| | | Fat | .9 | 7.1 |
| | II | Breast | 2.1 | 6.4 |
| | | Fat | .9 | 10.9 |
| Lactating rat I (7 days postpartum) | | Breast | 11.5 | 17 |
| | | Fat | 1.7 | 11 |
| | | Milk (fetus 1) | | 23 |
| | | ( " 2) | | 13 |

ing the latent period, further exposure of cells to the carcinogen produces enhanced effects. Our experiment shows that 3-methylcholanthrene is removed from the breast with passage of time, but a significant amount remains in these tissues for a prolonged period following a single feeding. The possibility that persistence of 3-methylcholanthrene in mammary glands may be important for neoplastic transformation has been clearly shown by Huggins' experiment in which a single feeding of 20 mg of 3-methylcholanthrene elicited mammary cancer in rats after a long latent period(2).

Thus, in chemical carcinogenesis of the rat breast, 2 factors seem to be involved: first, concentration of administered carcinogen in the target tissues; and second, persistence of the carcinogen in these tissues. Perhaps the adipose tissue of mammary gland functions as a storage depot of the carcinogen. Proximity of adipose tissue to the mammary gland tissue facilitates further exposure of mammary epithelial cells to the carcinogen, thus augmenting the neoplastic transformation. The vehicle, sesame oil, may also play an important role in transportation of fat-soluble carcinogens to breast and fat. Studies with aqueous suspensions of 3-methylcholanthrene are now being undertaken.

*Summary.* 3-methylcholanthrene in a single dose of 10 mg/ml sesame oil when fed to rats through a gastric tube, concentrated in the breast and fat of virgin female rats and in milk and breast of lactating rats. The level of 3-methylcholanthrene in other tissues was either very small or insignificant. It was further demonstrated that 3-methylcholanthrene persisted in a significant amount in these tissues at the end of 8 days following a single feeding of the carcinogen.

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# Absence of Hypoglycemia and Hypophosphatemia Following Initial Dosages of Phenethylbiguanide (D.B.I.)*† (25344)

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That phenethylbiguanide (D.B.I.) produces hypoglycemic effects in alloxanized animals, whereas sulfonylureas do not, suggests that the mechanisms of actions of these oral insulin substitutes are different(1). Ingestion of relatively large single dosages of tolbutamide or chlorpropamide regularly produces hypoglycemia and hypophosphatemia in most patients whose diabetes developed in adult years(2-5). In this study, D.B.I. was administered in single dosages to diabetics in amounts which, if given daily for 2 to 5 days lower blood glucose levels and improve glucose tolerance in the majority of diabetics(6). The results indicate that such a single dosage of D.B.I. initially has no effect upon blood sugar, serum inorganic phosphorus, glucose tolerance or insulin tolerance.

*Materials and methods.* All patients taking insulin were transferred to regular insulin at least 2 days earlier and the usual dosage was omitted the day of study. In each instance neither ketosis nor lowering of serum total $CO_2$ content was present. Except in control studies 150 mg of phenethylbiguanide were taken *per os* 45 minutes prior to serial hourly samplings of venous blood or before the glucose or insulin tolerance test. Blood sugar and serum inorganic phosphorus levels as we determined are given in Tables I and II and Figs. 1 and 2.

*Results.* There is no evidence that these amounts of phenethylbiguanide exerted hypoglycemic or hypophosphatemic effects. Changes in serial samplings, and in glucose and in insulin tolerance tests were the same with and without phenethylbiguanide and any differences which appeared were not statistically significant.

*Discussion.* Hall and colleagues(7) reported a significant fall in blood sugar in 60% of their diabetics following a single initial dosage of 150 mg of D.B.I., but control observations without the drug, which in our laboratory show a definite spontaneous hypoglycemic trend on serial sampling(2,3), were not made. In our studies, phenethylbiguanide or D.B.I. administered to diabetics in a single dosage of 150 mg, a quantity which when continued on daily basis for 2 to 5 days regulates levels of glucose in the majority of diabetics(6), failed to exert either a hypoglyce-

TABLE I. Absence of Any Effect of DBI in Single Dosage, 150 mg at Minus 45 Minutes, in Venous Blood Sugar and Serum Inorganic Phosphorus of Diabetics.

| Hr | Without DBI<br>Mean change* ± S.D. (mg %) | (#) | With DBI<br>Mean change ± S.D. (mg %) | (#) |
|---|---|---|---|---|
| | Blood sugar | | | |
| 1 | − 3 ± 17 | (16) | − 7 ± 20 | (16) |
| 2 | − 9 ± 14 | ( " ) | −12 ± 20 | (15) |
| 3 | −25 ± 22 | ( " ) | −20 ± 26 | (16) |
| 4 | −30 ± 23 | ( " ) | −24 ± 31 | ( " ) |
| 5 | −33 ± 22 | (15) | −34 ± 41 | ( " ) |
| | Serum inorganic phosphorus | | | |
| 1 | +.02 ± .17 | (15) | −.02 ± .30 | (16) |
| 2 | +.01 ± .17 | ( " ) | +.01 ± .62 | (15) |
| 3 | +.04 ± .24 | (14) | +.03 ± .73 | (16) |
| 4 | +.17 ± .28 | ( " ) | +.04 ± .72 | ( " ) |
| 5 | +.15 ± .33 | (15) | +.17 ± .74 | (15) |

* From values at zero time.

TABLE II. Serum Inorganic Phosphorus Changes without and with DBI during Glucose Tolerance Tests and Insulin Tolerance Tests in Diabetes.

| Time (min.) | Without DBI<br>Mean change* ± S.D. (mg %) | (#) | With DBI<br>Mean change ± S.D. (mg %) | (#) |
|---|---|---|---|---|
| | Glucose tolerance test | | | |
| 30 | +.15 ± .70 | (9) | +.01 ± .17 | (8) |
| 60 | −.19 ± .25 | (") | +.05 ± .18 | (10) |
| 90 | −.21 ± .31 | (") | +.04 ± .28 | ( " ) |
| 120 | −.20 ± .25 | (8) | −.01 ± .36 | (9) |
| | Insulin tolerance test | | | |
| 30 | −.49 ± .24 | (11) | −.66 ± .33 | (12) |
| 60 | −.46 ± .54 | ( " ) | −.34 ± .58 | (13) |
| 90 | −.37 ± .50 | ( " ) | −.43 ± .45 | (10) |
| 120 | −.01 ± .60 | ( " ) | −.42 ± .34 | (11) |

* From values at zero time.

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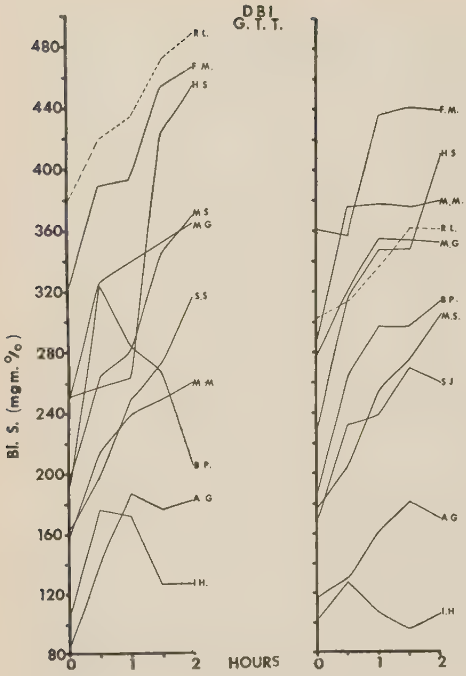
* Supported by grants from John A. Hartford Fn. and U. S. Dept. of H.E.W.

† N′-β-phenethylformamidinyliminourea hydrochloride.



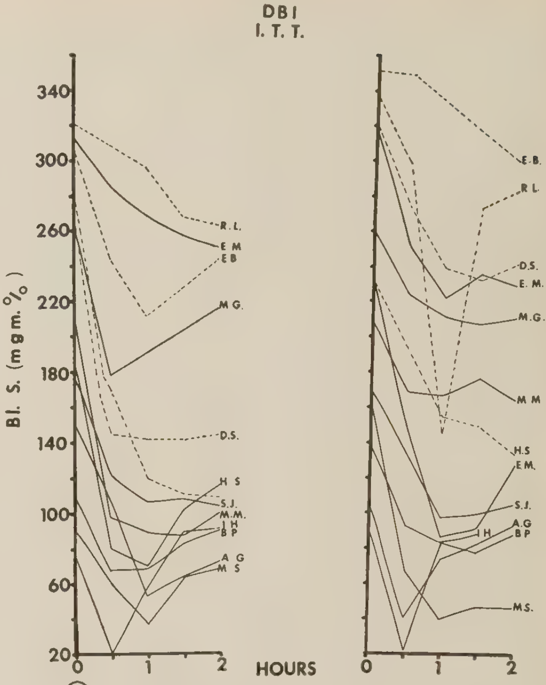


FIG. 1. Glucose tolerance tests without and with phenethylbiguanide (D.B.I.). Curves on left represent blood sugar levels in control glucose tolerance studies; repeat of tolerances after single dosage of D.B.I. (150 mg *per se*) at minus 45 min. are shown on right. Broken line identifies juvenile onset diabetes; solid lines refer to adult type diabetes. Mean changes from zero time in venous blood sugar in mg % following 1.75 g of glucose/kg body wt at zero time:

| | 30 min. | 60 min. | 90 min. | 120 min. | |
|---|---|---|---|---|---|
| Without D.B.I. | 69 ± 29 | 75 ± 28 | 107 ± 45 | 116 ± 61 | These differences are not statistically significant. |
| With D.B.I. | 39 ± 38 | 67 ± 48 | 76 ± 53 | 85 ± 66 | |

FIG. 2. Insulin tolerance tests without and with phenethylbiguanide (D.B.I.). Results of insulin tolerance tests without D.B.I. are shown on left; those following 150 mg of D.B.I. are on the right. Broken lines identify juvenile onset diabetes. Commercial crystalline insulin, 0.1 u/kg body wt was inj. intrav. at zero time. Mean changes in blood sugar in mg % from values at zero time:

| | 30 min. | 60 min. | 90 min. | 120 min. | |
|---|---|---|---|---|---|
| Without D.B.I. | −71 ± 37 | −85 ± 44 | −78 ± 47 | −72 ± 46 | These differences are not statistically significant. |
| With D.B.I. | −52 ± 25 | −77 ± 53 | −66 ± 39 | −68 ± 31 | |

mic or hypophosphatemic effect. In these respects, D.B.I. differs from sulfonylureas such as tolbutamide or chlorpropamide which on initial exposure do lower the levels of blood sugar and of serum inorganic phosphorus. However, in most trials with sulfonylureas, larger quantities were administered. This was not possible with D.B.I. because of gastrointestinal effects.

The results of glucose tolerance tests and of insulin tolerance tests support the findings obtained during serial measurements of blood sugar and of inorganic phosphorus for 5 hours after 150 mg of D.B.I. Again there is no evidence of a hypoglycemic or a hypophosphatemic effect.

This lack of response to initial dosages of D.B.I. coupled with ultimate effectiveness if therapy is continued, points to a cumulative effect, perhaps related to increasing levels of the drug or tissue responsiveness which may develop without accumulation.

*Summary and conclusion.* 1. Phenethylbiguanide (D.B.I.) in a single dose of 150 mg failed to induce a decrease in blood sugar or in serum inorganic phosphorus during succeeding 5 hour period in diabetic patients previously controlled with or without insulin.

2. This amount did not affect blood sugar and serum inorganic phosphorus changes during glucose tolerance tests and insulin tolerance tests in the same subjects. 3. Since these amounts of D.B.I. when continued on daily dosage do induce hypoglycemia, it is evident that such responses result either from accumulation of the drug in body fluids and tissues, represent acquired responsiveness upon continued exposure, or both.

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## Duration of Progestational Effect in Rabbit Endometrium after Prolonged Administration of Progestogens.* (25345)

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Since the study by De Fremery, Luchs and Tausk(1), it has generally been assumed that progestative effect of progesterone on rabbit endometrium cannot be extended beyond 17 days. This could be explained by interaction of 3 factors: 1) lack of response of endometrium after 17 days; 2) lack of estrogen, the primary effect of which is considered necessary for progestative transformation and its continuity; 3) suppression of pituitary gonadotropins or a similar factor which might stimulate endogenous estrogen or progesterone formation. The complexity of the factors involved led us(1) to assume the existence of an "inhibitory mechanism which needs further elucidation." This paper is devoted to the above problem.

*Technic.* Experiments were carried out on 200 female rabbits weighing approximately 1,000 g each. They were primed by daily injections of 15 $\mu$g estrone (in 0.15 ml olive oil) for 6 days. After 1 day's rest, the progestational compounds were injected subcutaneously at dosage level of 0.5 mg (in 0.05 ml olive oil) daily. The progestational effect was assessed according to McPhail(2) after sacrificing rabbits within 1-80 days after commencement of injection of the progestational substance. At autopsy, 2 segments were removed from each horn and preserved in 4% formalin (1:10) for histological examination. Evaluation of progestational transformation was carried out according to McPhail(2), the sections of uterus scored as 0, 1, 2, 3, 4 respectively. Furthermore, ovaries were inspected to ensure that no corpora lutea were present. We did not use the technic of De Fremery, Luchs and Tausk(1) who followed progestational transformation in each animal by laparotomy and removal of a piece of uterine horn at different time intervals, lest it interfere with degree and continuity of progestational transformation.

*Results.* A. *Progesterone.* Table I shows that, during 80 days' treatment with progesterone, secondary transformation of endometrium could be obtained. We believe that this is possible after longer intervals; thus, in

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* We thank Prof. M. Tausk, for progesterone (Progestine-Organon), Prof. K. Junkmann for hydroxy-progesterone caproate (Proluton Depot-Schering), Dr. L. J. Piccoli for norethindrone (Norlutin-Parke, Davis & Co., Dr. J. K. McGowan for methallyl-nortestosterone SC-9022-Searle & Co. This study was generously aided by grant from Dr. W. M. Lyons of Winnipeg.

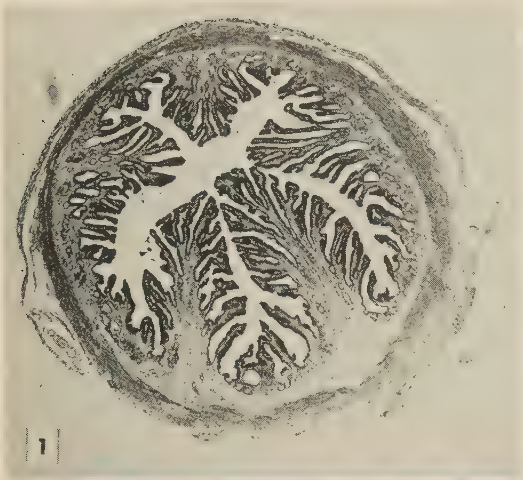
one rabbit treated for 96 days, degree 4 of progestational transformation was found (Fig. 1).

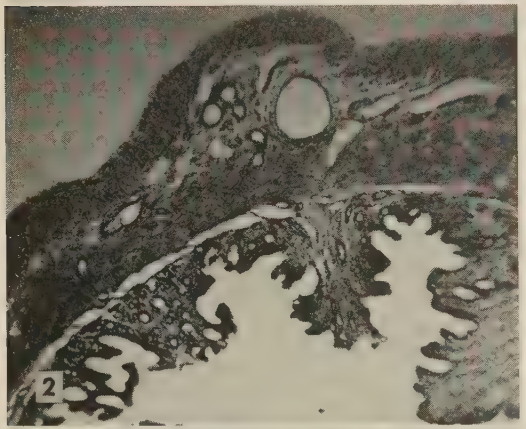
Table I shows, however, that at certain periods there appear negative or low score progestative phases. These periods are 15th-19th day and multiples thereof, *i.e.*, 29th-35th day, etc. This can only be explained by the breaking down of progestational phase at these terms and its immediate restoration.

We studied this problem in 2 ways: 1) Vaginal smears were full of erythrocytes and debris during days of progestational breakdown, whereas during remaining time they showed an epithelial pattern. In the former, the uterus showed decay of the progestational phase (Fig. 2). In Fig. 3 the transient proliferative stage, assumed to occur on day after vaginal bleeding, is presented. This photograph was taken from uterus of a rabbit on 34th day of protracted progesterone treatment.

2) We also studied how long the endometrial breakdown could last, and how soon the progestational phase could be restored. The vaginal smears continued to show erythrocytes and debris only during 2 consecutive days. As a rule we obtained the progestational phase in estrogen-primed rabbits as early as 2 days after injection of progesterone (*cf.* Table I). It can thus be safely said that during protracted progesterone treatment a breakdown of progestational endometrium occurs on 17th ± 2 day, followed within 2 days by building up of a new progestational endometrium.

B. *New Progestogens.* We tested the following new progestogenic substances: 17-alpha-hydroxy-progesterone caproate, norethindrone, methallyl-nortestosterone, at 0.5 mg/day. After estrogen-priming rabbits received the different progestogens for 20, 40, 60 and 80 days, respectively. At intervals of 17 ± 2 days vaginal smears showed erythrocytes and debris, indicating breakthrough bleeding. In all cases a progestational endometrium was found at necropsy. An endometrium of 60th





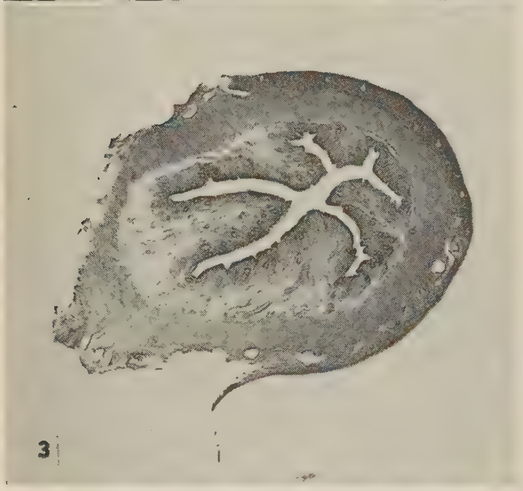


FIG. 1. Rabbit uterus (25×). Progestational transformation after 96 days' treatment.

FIG. 2. Uterus of rabbit on 17th day of protracted treatment with 0.5 mg/day progesterone (40×). Note decay of progestational phase.

FIG. 3. Uterus of rabbit on 34th day of protracted treatment with 0.5 mg/day progesterone (30×). Note absence of progestational transformation.

TABLE I. Progestational Effect in Rabbits after 1-80 Days' Subcutaneous Treatment with 0.5 mg Progesterone.

| Days of treatment | Progestational scoring | Days of treatment | Progestational scoring | Days of treatment | Progestational scoring | Days of treatment | Progestational scoring |
|---|---|---|---|---|---|---|---|
| 1 | 0, 0 | 21 | 2 | 41 | 3, 3 | 61 | 0, 0 |
| 2 | 3, 4 | 22 | 1, 3 | 42 | 3, 3 | 62 | 0, 1 |
| 3 | 4 | 23 | 1, 2, 2, 2, 3 | 43 | 3, 4 | 63 | 0, 2 |
| 4 | 4 | 24 | 1, 3 | 44 | 4, 4 | 64 | 0, 1, 2 |
| 5 | 4 | 25 | 1, 3 | 45 | 0, 0, 1 | 65 | 0, 2 |
| 6 | 4 | 26 | 2, 4 | 46 | 0, 0, 1, 1 | 66 | 0, 4 |
| 7 | 4 | 27 | 3, 4 | 47 | 0, 0, 1 | 67 | 0, 2 |
| 8 | 4 | 28 | 4, 4 | 48 | 0, 0, 2 | 68 | 0, 3 |
| 9 | 4 | 29 | 0, 0, 1, 1, 2 | 49 | 0, 1, 1 | 69 | 2, 4 |
| 10 | 4 | 30 | 0, 1, 1, 1 | 50 | 0, 1, 1 | 70 | 4, 4 |
| 11 | 4 | 31 | 0, 0, 1, 1 | 51 | 0, 0, 1, 4 | 71 | 3, 4 |
| 12 | 3 | 32 | 0, 0, 1, 2 | 52 | 2, 3 | 72 | 4, 4 |
| 13 | 4, 2 | 33 | 0, 0, 1, 2 | 53 | 2, 4 | 73 | 4, 4 |
| 14 | 4, 4 | 34 | 0, 0, 1, 3 | 54 | 3, 4 | 74 | 1, 2 |
| 15 | 0, 1, 2, 3 | 35 | 0, 0, 1, 2 | 55 | 4, 4, 4 | 75 | 0, 4 |
| 16 | 0, 1, 2, 4 | 36 | 3, 4 | 56 | 3, 4 | 76 | 0, 1 |
| 17 | 0, 1, 3, 3 | 37 | 2, 2 | 57 | 2, 3, 4 | 77 | 0, 2 |
| 18 | 2, 3, 3, 3 | 38 | 2, 4 | 58 | 4, 4 | 78 | 0, 1 |
| 19 | 2, 3, 3 | 39 | 3, 4 | 59 | 2, 4 | 79 | 0, 3 |
| 20 | 4, 4 | 40 | 2, 2 | 60 | 0, 1, 2 | 80 | 4, 4 |

day showed beginning of breakthrough bleeding as expected.

*Discussion.* Our results confirm reports of earlier investigators(1) that the progestative phase of rabbit endometrium breaks down after 17 days in spite of continued treatment. On the other hand, they reveal the almost forgotten fact that in the rabbit transformation to the second generative phase is accomplished within 48 hours. Therefore the progestational phase which breaks down near the 17th ± 2 day is restored within 48 hours when treatment with progestogens is continued. This would mean that through protracted treatment with progestogens, rabbit endometrium can be kept indefinitely in the second phase.

Junkmann(3,4) also noted waning of the progestational effect of a single injection of 20 mg hydroxy-progesterone caproate between 15th and 19th day. He believed that daily addition of 0.05 $\mu$g estradiol prolonged the progestational effect.

The progestational effect obtained by protracted treatment of rabbits with chorionic gonadotropin is likewise restricted to about 17 days' duration(5). By resuming gonadotropin treatment beyond this term it is, however, possible to obtain a quick return of the second phase(6,7). The mechanism of this phenomenon was studied by Zondek(8,9) who found that breakthrough bleeding might be obtained even after 5 days' treatment and that it closely resembled human menstruation.

In women the problem is different, as breakthrough bleeding occurs with progesterone(10, 11) and with most of the new progestogens. Greenblatt *et al.*,(12,13) delayed menses for as long as 6-7 months with 20-30 mg norethisterone daily. Large doses (up to 1,000 mg/day) of ethisterone, 17-acetoxyprogesterone, 17-hydroxyprogesterone caproate, could not prevent breakthrough bleeding. Boschann (14) delayed breakthrough bleeding until the 28th-42nd day of treatment with 100-200 mg/day ethinyl nortestosterone. Similar results are obtainable with medroxy-progesterone acetate. Following protracted treatment with chorionic gonadotropin, breakthrough bleeding occurs also between 17th and 19th day(15).

*Summary.* Continued injection of rabbits with progesterone, 17-alpha-hydroxy-progesterone caproate, norethindrone and methallyl-nortestosterone, maintained a progestative phase for 80 days and longer. At intervals of 17 ± 2 days, however, there occurred breakthrough bleeding followed by renewal of second phase within 2 days.

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## Effect of $Sr^{89}$ on Fluoride Retention in the Rat.* (25346)

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Increasing numbers of communities using fluoridated water along with the increasing amount of radioactive strontium in the atmosphere have caused considerable speculation whether the mutual presence of fluoride and strontium increases retention of each within the body(1). The purpose of this study is to investigate retention of fluoride and radioactive strontium when both are fed simultaneously in the rat.

*Method.* One mc of $Sr^{89}$ (as strontium chloride) was diluted to one liter with fluoride-free redistilled water. This stock solution was used throughout the 3 series of experiments. Series I. A total of 24 male Sprague-Dawley rats was divided according to initial body weight into 4 groups of 6 animals each. All animals received a low-fluoride diet (0.05 $\mu$g F/g) and fluoride-free redistilled drinking water *ad lib.*, and were housed in an air-conditioned room in individual stainless steel metabolism cages. Animals in Group A served as controls and received no additional supplement. Those in Group B received a single dose of 0.5 ml of $Sr^{89}$ stock solution (501,600 cpm/ml) by stomach tube. Animals in Group C received 0.15 mg F (as NaF) daily by stomach tube, and those in Group D received both a single initial dose of 0.5 ml $Sr^{89}$ and 0.15 mg F daily. Duration of experiment was 10 days. Urine and feces samples were collected separately throughout each 24-hour period. One-half of the urine and feces samples, was analyzed for F by methods previously described (2). The remaining half was analyzed for strontium by charring samples under infrared lamps and ashing for 6 hours in muffle furnace at 600°C. The ash was dissolved in 2 ml 0.1 N HCl, transferred to volumetric flask and diluted with redistilled fluoride-free water to 10 ml. A 1 ml aliquot was placed in an aluminum planchet and evaporated to dryness at 45°C and activity determined with a scintillation counter. Carcasses and femurs were analyzed for F by methods previously described(2). To determine strontium content in calcified tissues, an aliquot of ash was placed in aluminum planchets, dissolved in 2 ml 0.1 N HCl, evaporated to dryness, and the activity counted. Series II was identical to Series I except for amounts of supplemental F and $Sr^{89}$ (Table III) and duration of experiment (20 days). Series III was similar to Series I and II except that its duration was 100 days and supplements were different (Table V).

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* This study supported in part by grant from U.S.P.H.S.

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TABLE I. Comparison of Fluoride and Strontium Retention in Carcass and Femur of the Rat. Series I animals

| Group | Wt gain (g) | Carcass fluoride µg/g | Carcass fluoride % ret.* | Femur fluoride µg/g | Femur fluoride % ret.* | $Sr^{89}$ analysis (cpm/g ash) Carcass | $Sr^{89}$ analysis (cpm/g ash) Femur |
|---|---|---|---|---|---|---|---|
| A | 21 | 66 ± 19 | | 69.9 ± 4.3 | | 146 ± 78 | 121 ± 84 |
| B | 23 | 57 ± 5 | | 60.5 ± 11.6 | | 18,800 ± 800 | 18,700 ± 1700 |
| C | 17 | 415 ± 28 | 67 | 585 ± 33 | 4.8 | 196 ± 65 | 165 ± 56 |
| D | 19 | 398 ± 11 | 69.7 | 550 ± 53 | 5.6 | 17,800 ± 900 | 18,600 ± 1900 |

A = controls; B = .5 ml $Sr^{89}$ (activity of 501,600 cpm/ml); C = .15 mg F; D = .5 ml $Sr^{89}$ plus .15 mg F.

* Based upon total F.

Results of F and strontium retention in Series I animals are found in Table I. When the femurs were used for evaluation of F retention, similar proportional findings to those in the carcass were found except that the amount of fluoride was less.

Excretion data obtained in Series I are found in Table II. Urine contained about 4 times as much fluoride as feces in controls, but about 9 times as much when 0.15 mg F was fed. Similar amounts of radioactivity were found in both feces and urine of control (Group A) and fluoride groups (Group C) due undoubtedly to background activity of the counter. When 0.5 ml of $Sr^{89}$ was administered 1912 cpm were found in feces and 2697 cpm when both F and $Sr^{89}$ were given.

TABLE II. Average Daily Excretion of Fluoride and Strontium$^{89}$ in the Feces and Urine. Series I animals

| Group | Feces µg F | Feces $Sr^{89}$ (cpm) | Urine µg F | Urine $Sr^{89}$ (cpm) |
|---|---|---|---|---|
| A | 1.15 | 61 | 4.10 | 58 |
| B | 1.63 | 1912 | 3.85 | 870 |
| C | 2.64 | 60 | 24.8 | 60 |
| D | 2.23 | 2697 | 25.2 | 729 |

A = controls; B = .5 ml $Sr^{89}$ (activ. of 501,600 cpm/ml); C = .15 mg F; D = .5 ml $Sr^{89}$ plus .15 mg F.

The F and strontium retention in Series II animals is found in Table III. No differences from controls in F retention were found in the carcass or femur of animals receiving $Sr^{89}$. When no strontium was administered, but 1 mg F was given daily, activity of carcass was greater than in controls. Significantly less activity was found in the carcass ($p = 0.01$) when both strontium and fluoride were administered than when similar amounts of strontium were given in the absence of F. Similar results were seen for the femur, for significantly less ($p = 0.0001$) activity was found in animals receiving both $Sr^{89}$ and F than in similar animals receiving the same amount of $Sr^{89}$ and no F.

Excretion data for animals in Series II are found in Table IV. In controls 6.6 µg F were found in feces and 18.4 µg in urine. In animals receiving $Sr^{89}$ but no fluoride there was 7.1 µg F in feces and 20.9 µg in urine, similar to values in control animals.

Amount of fluoride and strontium retained in the carcass and femur of the Series III animals is found in Table V.

*Discussion.* There was a significant difference ($p = 0.001$) in amount of fluoride retained in animals receiving $Sr^{89}$ in both whole carcass and femur when compared to similar animals receiving the same amount of fluoride

TABLE III. Comparison of Fluoride and Strontium Retention in Carcass and Femur of the Rat. Series II animals

| Group | Wt gain (g) | Carcass fluoride µg/g | Carcass fluoride % ret.* | Femur fluoride µg/g | Femur fluoride % ret.* | $Sr^{89}$ analysis (cpm/g ash) Carcass | $Sr^{89}$ analysis (cpm/g ash) Femur |
|---|---|---|---|---|---|---|---|
| A | 60 | 95.0 ± 18.6 | | 145 ± 17 | | 4,340 ± 960 | 9,900 ± 2,330 |
| B | 52 | 96.1 ± 8.4 | | 166 ± 15 | | 628,000 ± 20,900 | 821,000 ± 54,600 |
| C | 43 | 4410 ± 80 | 58.5 | 5860 ± 1840 | 2.6 | 9,300 ± 1,700 | 10,780 ± 1,680 |
| D | 28 | 5060 ± 200 | 61.5 | 5250 ± 990 | 2.2 | 562,000 ± 28,600 | 559,000 ± 31,200 |

A = controls; B = .5 ml $Sr^{89}$ (activity of 283,100 cpm/ml); C = 1.0 mg F; D = .5 ml $Sr^{89}$ plus 1.0 mg F.

* Based upon total F.

TABLE IV. Average Daily Excretion of Fluoride and $Sr^{89}$ in Feces and Urine.
Series II animals

| | Feces | | Urine | |
|---|---|---|---|---|
| Group | μg F | $Sr^{89}$ (cpm) | μg F | $Sr^{89}$ (cpm) |
| A | 6.6 | 351 | 18.4 | 219 |
| B | 7.1 | 6110 | 20.9 | 5570 |
| C | 24.6 | 404 | 248.0 | 210 |
| D | 41.1 | 7560 | 259.0 | 4610 |

A = controls; B = .5 ml $Sr^{89}$ (activ. of 283,100 cpm/ml); C = 1.0 mg F; D = .5 ml $Sr^{89}$ plus 1.0 mg F.

but no $Sr^{89}$. The reason for these reversals in fluoride retention is not known, except that in Series I animals the $Sr^{89}$ was given in one dose at beginning of study, while in Series II and III the $Sr^{89}$ was administered daily. It is reasonable to assume that this daily contact contaminated the animals with fluoride to the degree that there was increased retention of fluoride due to contamination and not due to a metabolic increase in fluoride retention resulting from a physiologic role of $Sr^{89}$ increasing fluoride absorption or retention, or both. This reasoning is substantiated by the results obtained in Group C and D animals in all 3 series, where there were no significant differences in fluoride retention in groups receiving fluoride alone (Group C) or both $Sr^{89}$ and fluoride combined (Group D). It thus appears that presence of $Sr^{89}$ is not associated with increased retention of fluoride in the rat.

The data comparing retention of strontium in presence of fluoride is of interest, since the data suggest that fluoride protects against strontium retention. In Series I animals, presence of fluoride given once at beginning of study resulted in no more $Sr^{89}$ retained in either the whole carcass or the femur than was found in absence of fluoride. In the study lasting 20 days (Series II) where both $Sr^{89}$ and fluoride were given daily there was significantly less ($p = 0.01$) activity in whole carcass and in femur in the group receiving both $Sr^{89}$ and fluoride than in the group receiving $Sr^{89}$ and no fluoride. In the 100 day study (Series III) retention of $Sr^{89}$ in presence of fluoride was not significantly different from that in animals receiving $Sr^{89}$ and no fluoride. More data are needed, however, before definite conclusions should be drawn as to protective effect of fluoride against strontium retention. In any event, the data demonstrate that fluoride does not *increase* retention of strontium in the body of the rat. Differences between carcass $Sr^{89}$ retention and skeletal $Sr^{89}$ retention in presence and absence of fluoride need further investigation also, especially in the light of recent evidence suggesting that certain nutrients may increase retention of fluoride in both soft tissue and skeleton(3,4).

Excretion data (Tables II and IV) also suggest that daily administration of fluoride tends to protect against $Sr^{89}$ accumulation in the skeleton. For example in animals receiving $Sr^{89}$ and fluoride for 10 days (Table II) there was an activity of 2,697 cpm in the feces (Group D), and 729 cpm in urine, whereas the group receiving identical amounts of $Sr^{89}$ but no fluoride (Group B) had significantly less ($p = 0.03$) activity in feces and significantly more in the urine ($p = 0.05$). This suggests that presence of fluoride renders $Sr^{89}$ less metabolically available. This is similarly confirmed by examining amount of $Sr^{89}$ activity in the carcass and femurs in these various groups. For example, there was an activity of 17,800 cpm in whole carcass of Series I animals receiving both $Sr^{89}$ and fluoride (Group D) but 18,800 cpm were found in animals receiving an identical amount of $Sr^{89}$ but no fluoride (Table I). Similar conclusions can

TABLE V. Comparison of Fluoride and Strontium Retention in Carcass and Femur of the Rat.
Series III animals

| | Wt gain | Carcass fluoride | | Femur fluoride | | $Sr^{89}$ analysis (cpm/g ash) | |
|---|---|---|---|---|---|---|---|
| Group | (g) | μg/g | % ret.* | μg/g | % ret.* | Carcass | Femur |
| A | 158 | 18.8 ± 1 | | 21.2 ± 1 | | .8 ± .35 | |
| B | 154 | 45.5 ± 4 | | 56.9 ± 2 | | 14,100 ± 399 | 17,800 ± 900 |
| C | 156 | 2370 ± 130 | 44.7 | 2810 ± 70 | 1.81 | 69.5 ± 6.3 | 647 ± 47 |
| D | 171 | 2240 ± 180 | 43.8 | 2810 ±127 | 1.92 | 15,400 ± 846 | 19,400 ± 998 |

A = controls; B = .5 ml $Sr^{89}$ (activity of 50,470 cpm/ml); C = .5 mg F; D = .5 ml $Sr^{89}$ plus .5 mg F.
* Based upon total F.

be drawn from Series II metabolism (Table IV) and retention (Table III) values.

While these conclusions seem to be an accurate evaluation of the data obtained, the long term effects of mutual feeding of both $Sr^{89}$ and fluoride under a variety of different conditions should be evaluated before making definite conclusions. It does appear, however, that presence of fluoride, at 3 different levels, is not associated with increased retention of $Sr^{89}$ in the body, nor, conversely, is the presence of $Sr^{89}$ associated with increased retention of fluoride in the body.

*Conclusions.* Administration of $Sr^{89}$ and fluoride together does not increase retention of either $Sr^{89}$ activity or fluoride in the whole carcass or femur of the rat. Preliminary evidence suggests, on the contrary, that presence of fluoride may decrease the amount of $Sr^{89}$ retained by the rat.

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## ECHO 11 Virus Associated with Aseptic Meningitis.* (25347)

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Several enteroviruses have clearly been associated with aseptic meningitis(1-3). Most prominent among this group are ECHO virus types 4, 6, 9, and 16; Coxsackie types A7 and A9, B1-5; and poliovirus types 1-3. Other types have been isolated from sporadic cases. Until the time of this study, ECHO 11 had been isolated only from normal children(4,5) and from sporadic cases of diarrhea(6). The present report is concerned with an outbreak of aseptic meningitis connected with this virus.

*History of outbreak.* During latter part of June and July, 1958, at least 7 children were admitted to Jefferson Davis Hospital with a febrile illness characterized by pleocytosis of the cerebrospinal fluid. In addition a mother of one of these children was admitted to the hospital as a "suspected case of poliomyelitis." The most common symptoms at time of admission were: frontal headache, vomiting, spiking fevers to 103°F, stiff neck and joints, acute abdominal pains, and occasional diarrhea and swollen cervical glands. Further physical examination did not show skin rash or mouth lesions. Duration of acute illness was 3 to 7 days. Hematological examination usually yielded a few immature blood cell forms. White blood cells, generally lymphocytes, in the cerebrospinal fluid ranged from 35 to 440/mm³. The diagnosis was aseptic meningitis, etiological agent unknown. Five of the 7 children lived in a low rental housing project of 1000 dwelling units for low income families. The majority of units are rows of 2-story apartments. The buildings are constructed of concrete with adequate bathroom and cooking facilities in each family unit. Occasional inspection by the administration keeps the living quarters in good repair. Insect control is part of this service. There are ample playground facilities for children. Visiting among neighbors in adjacent homes is very common, especially among children. It is not unusual for the Public Health Nurse to visit each family periodically to check on communicable diseases. A vaccination program is carried out through the hospital clinic and most children visited had received vaccines against diphtheria, whooping-cough, poliomyelitis, typhoid, and tetanus. *Field study.* Because of ease of collection and handling, it was decided to obtain only rectal swabs. Specimens were collected in 3 ml of M-H medium(7) containing a 10-fold concentration of antibiotics; swabs were stored on dry ice, and kept frozen in laboratory until tested. Thirty-six families, contributing 157 single specimens, were studied for symptoms and for virus. The criteria for choosing families were as follows. Specimens were obtained from 18 families who either reported a child with a recent history of admission to the hospital with presumptive diagnosis of aseptic meningitis, or had close contact with such a child. Samples were taken from affected child, from each available member of his family, and from members of contact families. As cases were limited to one side of the housing project, a control population of 18 families was selected from the opposite side of the courts. Although it was possible for older children to have mingled through community projects, the vast majority of children in this group were too young for such social admixture and their associations were generally limited to adjacent homes. Families in the control group were randomly selected from among larger families.

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* This study was aided by grant from National Fn.

*Laboratory methods.* Monkey kidney tissue cultures were used throughout the isolation and identification process(7). Swab suspensions were thawed at room temperature, centrifuged at 2000 rpm, and 0.1 ml of the supernate inoculated into each of 3 culture tubes. Inoculated cultures were incubated at 37°C and examined for cytopathic effects (CPE) until fifteenth day. If CPE occurred the tissue culture was harvested by freezing and thawing to liberate the cell-associated virus. If culture was harvested after sixth day of incubation, it was passaged through another tissue culture before titration. A calculated dose of 100 $TCD_{50}$ was used in all neutralization tests. As a rule the virus was first typed against polio antisera, and if not neutralized, further typings were carried out by Coxsackie and ECHO combination antiserum pools as described by Lim and Benyesh-Melnick(8). Confirmation was made by typing with antiserum indicated in results of the combination pool tests, both by neutralization and (for many of the strains) by complement fixation test.[†]

*Results.* Of the 36 families tested for presence of enteroviruses, 27 yielded positive isolations, 16/18 (89%) in the study group and 11/18 (61%) in control group. As shown in Table I, of 74 specimens in the study group, 33 were positive (45%) in contrast to 19 positive isolations from 83 specimens (23%) in the control group, the difference being significant ($P<0.01$).

TABLE I. Viral Isolation in Test and Control Series.

| Group | No. of families | No. of specimens | No. of isolations: Total | No. of isolations: ECHO 11 | No. of isolations: Other |
|---|---|---|---|---|---|
| Study | 18 | 74 | 33 | 19 | 14* |
| Control | 18 | 83 | 19 | 5 | 14† |

* There were 7 isolations of ECHO 4, 1 of ECHO 6, 1 of ECHO 9, 2 of polio 1, and 3 of polio 3.

† There were 3 isolations of ECHO 5, 4 of ECHO 13, 2 of ECHO 15, 2 of Coxsackie B2, 2 of Coxsackie B3, and 1 of Coxsackie B5.

The most prevalent virus isolated in the study group was ECHO 11, 19 of 33 isolations; the same agent was found among viruses isolated from control children, but to a lesser extent (5 of 19 isolations). Of the 12 families in the study group in which individuals were excreting ECHO 11 virus, 8 were infected with this virus alone. Three families were infected with both ECHO 11 and ECHO 4, and one family with ECHO 11 and ECHO 6.

Of 5 children and one mother admitted to the hospital with symptoms of aseptic meningitis, 2 were from families yielding ECHO 11 alone, one from an ECHO 4 family, one from a family infected with both ECHO 4 and ECHO 11, and one from a family infected with ECHO 9 and poliovirus type 1. Virus from the patient was of the type isolated from the family, except for the ECHO 9 patient. In this instance, ECHO 9 was isolated from patient's cerebrospinal fluid and feces, but 2 weeks later when family specimens were taken, 2 siblings were carrying poliovirus type 1. Spinal fluid was tested from 2 ECHO 11 patients but no virus was isolated.

The number of individuals with symptoms in the study group was 61% (45/74) in contrast to only 18% (15/83) in control series (Table II). In the study group, ECHO 11 was isolated from 22% (10/45) of individuals with symptoms but inapparent infection was common, the same virus being isolated from

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[†] The CF tests were kindly carried out by Dr. M. Shingu, Research Fellow in this laboratory.

TABLE II. Total Population Studied in Relation to Illness and Isolations of ECHO 11 and Other Enteroviruses.

| | Total No. individuals | With symptoms* | | | Without symptoms | | |
|---|---|---|---|---|---|---|---|
| | | Total | ECHO 11 | Other† | Total | ECHO 11 | Other† |
| Study | 74 | 45 | 10 | 10 | 29 | 9 | 4 |
| Control | 83 | 15 | 2 | 3 | 68 | 3 | 11 |

* These included any of the following, usually being multiple in a single patient: headache, fever and malaise, stiff neck, backache, muscle pains, sore throat, abdominal pain, vomiting, nausea, anorexia, diarrhea, swollen glands.

† See footnote to Table I.

31% (9/29) of asymptomatic patients. Of 19 individuals in the study group excreting ECHO 11 virus 53% had symptoms, the more frequent complaints being headache, fever, malaise, vomiting, abdominal pain, sore throat and stiff neck.

In the control group, ECHO 11 was isolated less often; only 5 individuals in 3 families yielded the virus. Only one of these families reported an illness and in the 3 members tested the virus was found. In 2 of these individuals the only symptom was a recent attack of diarrhea, whereas in the study group this symptom was not associated with isolation of ECHO 11. In the other 2 cases, ECHO 11 was found to be excreted together with Coxsackie B2, but in neither case were any symptoms reported.

Symptoms accompanied ECHO 11 infection in older children more frequently than in those under 5 years of age. In those under 5 years of age, only 1 of 7 virus carriers became ill, whereas 6 of 7 children over 9 years old had symptoms similar to those described above (Table III).

*Discussion.* Studies of aseptic meningitis outbreaks have usually shown that although there is a predominant virus associated with the disease, other enteroviruses may be concurrently isolated from individuals associated with the epidemic(1-3). This study has again revealed that it is possible for overlapping infections of many enteroviruses to occur in a limited area at the same time, making it difficult from a single isolation to determine the etiological relationship of the agent to the outbreak. The association of ECHO 11 with this epidemic is noteworthy. In comparing test and control populations, there was a higher degree of illness compatible with aseptic meningitis reported among individuals in the former than in the latter group (P<0.01). The symptomatology associated with these cases appeared more varied than that usually found in "classical" aseptic meningitis outbreaks.

TABLE III. Occurrence of Symptoms According to Age in Children of Study Group Excreting ECHO 11.

| | Age group (yr): | | | | |
|---|---|---|---|---|---|
| | 0-4 | 5-9 | 10-15 | >15 | Total |
| ECHO 11 with symptoms | 1 | 3 | 5 | 1 | 10 |
| ECHO 11 without symptoms | 6 | 2 | 1 | 0 | 9 |
| Total | 7 | 5 | 6 | 1 | 19 |

*Summary.* In a study of an outbreak of aseptic meningitis, ECHO 11 was the most prevalent virus isolated (24 of 52 isolations). Other enteroviruses associated with cases in the outbreak but to a lesser degree were ECHO 4 and polioviruses 1 and 3. Children over 5 years of age infected with ECHO 11 were more apt to have clinical symptoms of aseptic meningitis than younger children.

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# Activity Rhythm and Breast Cancer in Pituitary-Isografted C and $D_8$ Mice; Effect of Hypothalamus.[*] (25348)

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Mühlbock and Boot have shown that the estrous cycle of mice can be significantly altered by pituitary isografts(1,2).[†] Estrous, in turn, in the rodent, is locked in defined phase relations with 24-hour rhythms, such as that in body activity(3,4). Are the latter rhythms biologically more basic, however, and do they persist under conditions which alter or obliterate estrous(4,5)? It was of interest, then, to record gross motor activity and to find that a 24-hour periodic activity-pattern persists after pituitary isografting. In the same study, mammary carcinogenesis from pituitary isografts was analyzed and experimentally modified.

*Materials and methods.* Virgin female recipient and donor mice of 2 strains were used. The strains had been maintained by brother-to-sister mating for over 15 years and differed in that the C's (Bagg albino) had no demonstrable mammary tumor agent (MTA), while the $D_8$'s had a demonstrable MTA. From weaning and throughout the study, the mice had Purina Fox Chow and tap water continuously available. They were kept under natural conditions of illumination, in wooden cages with a wire mesh top, 5 mice in an 11″ x 5¾″ x 5¼″ space. Beginning during second month of life and continuing 7, 8, 14, or 16 times (at one-week intervals, or exceptionally, at 2-week intervals), recipient mice were given subcutaneously by trocar one of the following treatments: Pools of either 5 (I) or 3 (II) pituitaries, or one (III) hypothalamus with 3 pituitaries, or one (IV) hypothalamus alone, or 5 (V) or 3 (VI) pieces of femoral muscle. The 6 C-recipient groups and 2 $D_8$ recipient groups (I and V) consisted of 10 mice each. The other 4 $D_8$ recipient groups consisted of 5 mice each. Right and left axilla were used in alternation as sites of consecutive treatments. Age of donors (of same stock as recipients) varied from 3 to 5 weeks. Transplantations were done with assembly-line procedures immediately after killing donors by cervical dislocation by day (08-16). Fig. 1 shows area removed as "hypothalamus"[‡] as well as pituitary. Cancer rate and age were evaluated by inspection and palpation, the diagnosis verified on biopsy or autopsy material stained with hematoxylin-eosin and, frequently, also by tests of transplantability. Two of 40 $D_8$ recipients and 6 of 60 C recipients died non-cancerous before one year of age. Cancer incidence comparisons were by Fisher's exact test (6) with and without omission of deaths prior to one year of age, with similar results. During second year of life gross motor activity was measured concomitantly on 10 non-cancerous C mice from different experimental groups (including animals which developed later verified breast cancer). Activity was recorded for several weeks on each mouse, in individual electronic actometers(4,7) kept at 24°C in light daily from 06 to 18 hrs.

*Results.* Activity counts of individual C mice which had received different treatments, in each case, were on the average higher during daily dark periods than during light periods (*cf.* Fig. 2). Persistence of a 24-hour activity rhythm synchronized with the regimen of light and dark seems established for mice bearing pituitary isografts, in which pseudopregnancies reportedly replace normal estrous cycle(1,2). If periodic changes underlying activity rhythm, *e.g.*, in blood corticoids (8,9), also are synchronized with lighting

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[*] Supported by Elsa U. Pardee Fn., Amer. Cancer Soc., U.S.P.H.S., and Dept. of Public Welfare, State of Minn.

[†] Drs. Mühlbock and Boot discussed activity-rhythm measurements in pituitary-isografted mice with one of the authors (FH) and allowed him early insight into extensive and valuable data on estrous behavior in such mice.

[‡] Authors are indebted to Dr. Berry Campbell, Univ. of California at Los Angeles, for advice regarding hypothalamus removal.

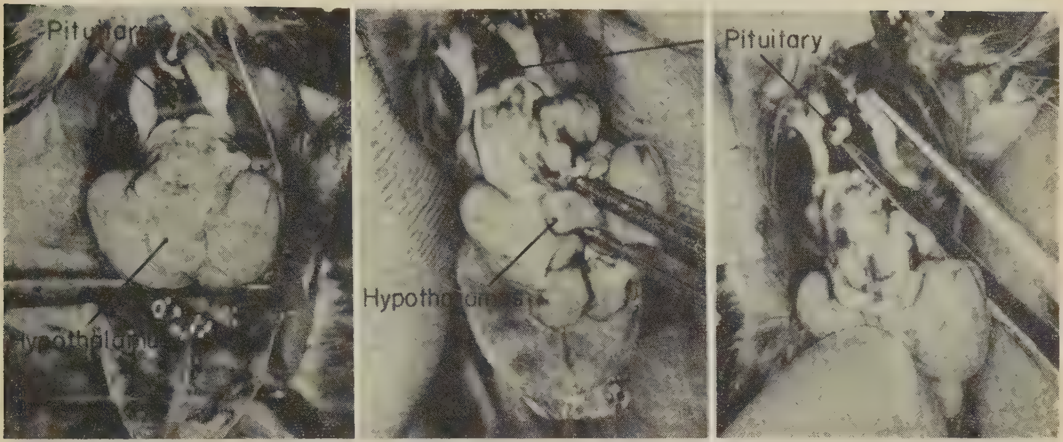


FIG. 1. Tissue removal from donors (see text).

schedule in mice bearing pituitary isografts, the data suggest persistence of the adrenal cycle(4,5,8-10) under conditions which significantly alter the estrous cycle.

In addition to changes with time, overall-activity levels differed from mouse to mouse. Similar differences existed, however, among individual mice from the same treatment-group (and were recorded earlier on untreated mice), although the actometers had been calibrated before use. Further work is needed to explore possible differences among experimental groups in 1) amplitude or level (rather than period) of light-dark synchronized activity rhythm or in 2) behavior of period of rhythm in the absence of such synchronization.

Breast cancer development in some groups given pituitary isografts is shown in Fig. 3 (with corresponding data on mice given muscle). The upper sections show data on $D_8$ mice with MTA: The number of mice with breast cancer was 1 of 15 $D_8$'s given muscle [0 of 5 $D_8$'s given hypothalamus (not graphed)], as compared to 13 of 15 $D_8$'s given pituitaries (all maintained as virgins). Fig. 3 (bottom) also shows data on virgin C

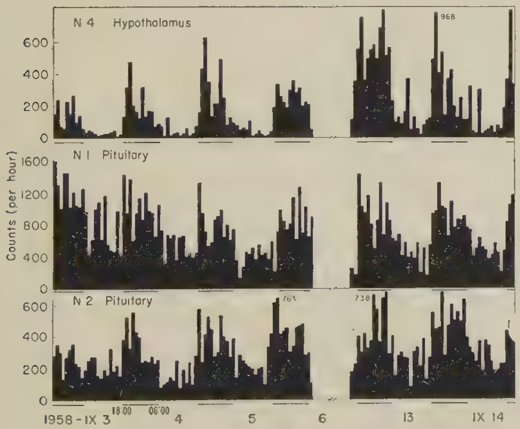
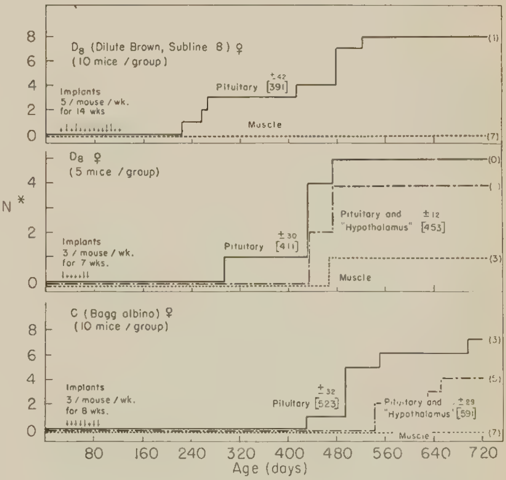


FIG. 2. Light-dark synchronized activity rhythm whether or not pituitaries were isografted. Activity in arbitrary units is higher during dark periods (above horizontal bars).



*N = No. of mice with breast cancer (adenocarcinomata and adenoacanthomata)
No. dead without breast cancer, ... ( ) Mean cancer age ,.. [ ] ± S.E.

FIG. 3. Breast cancer incidence in 65 of recipients studied—work involving also 1390 donors of corresponding inbred stock, sex, and age. Incidence in virgin $D_8$ mice is low, whether or not muscle treatments are given; it is significantly raised by pituitary isografts. In non-cancerous C mice pituitary isografts induce breast cancer, though at a more advanced cancer age than in $D_8$'s. Insertion of hypothalamus at times of pituitary isografting delays mammary carcinogenesis.

TABLE I. Body Weights of Mice without Cancer, at About 16 Months of Age.*

| | C | | $D_8$ | |
|---|---|---|---|---|
| Treatment | No. of inserts (No. of mice) | Body wt, g | No. of inserts (No. of mice) | Body wt, g |
| Muscle | 16 × 4 (5) | 32.0 ± 1.5† | 14 × 5 (7) | 25.4 ± .4† |
| ” | 8 × 3 (8) | 29.6 ± .7 | 7 × 3 (5) | 32.2 ± 1.0 |
| Hypothalamus | ” (8) | 31.4 ± .7 | ” (5) | 32.6 ± .2 |
| Pituitary | 16 × 4 (3) | 29.0 ± 2.0 | 14 × 5 | ‡ |
| ” | 8 × 3 (5) | 31.8 ± 1.0 | 7 × 3 | ‡ |
| ” & hypothalamus | ” (7) | 33.0 ± 1.1 | ” (2) | 28.0 ± .0 |

* See text. † Mean ± S.E. ‡ Those alive all with breast cancer.

mice, without demonstrable MTA, revealing again the carcinogenic effect of pituitary isografts, in agreement with results on other stocks without demonstrable MTA(2), recently reviewed(1). Differences in cancer incidence between muscle and pituitary treatment-groups are significant for each stock: The P values for the 2 available comparisons on $D_8$ mice were $<.01$ and $<.03$ respectively, and $<.01$ for the C mice.

Breast cancers recorded were adenoacanthomata or adenocarcinomata, both types being found in C's as well as $D_8$'s with pituitary isografts. Squamous metaplasia was seen more often, however, in C mice without MTA than in $D_8$ mice with MTA, in agreement with results of earlier work on other stocks(11).

The carcinogenic effect of pituitary isografts (Fig. 3) is in close agreement with earlier work(1,2,12,13); its significance is underlined by control of secular variation, since all treatment groups compared were set up concomitantly and by control of experimental procedures, since the standards of reference are data from mice given muscle rather than data from colony at large, a procedure previously used, which does not allow for control of trauma (*e.g.*, from trocar puncture or from "irritation" by inserted tissues). Control of exposure to experimental procedures was sought since trauma might affect carcinogenesis, although some data supporting this suggestion were not statistically significant(14). In this study at 16 months of age, body weight of $D_8$ mice given 14 muscle treatments was lower than that in mice given only 7 such treatments, but this possible effect of trauma was not apparent in comparison of C mice given 8 or 16 treatments (Table I). Herein trauma, as such, was not studied, only its possible effect was controlled. The contribution of pituitary isografts themselves to breast cancer development in the mouse stands out more significantly, whether or not a demonstrable MTA is present.

*Stock and number of implants in relation to breast cancer incidence and cancer age.* Although cancer developed in mice without (C) as well as with MTA ($D_8$), the question might be raised whether presence of agent enhanced carcinogenesis (*cf.* 1,2). Cancer incidence in C mice given totals of either 80 or 24 pituitaries was 7 of 10 in each group while for the $D_8$'s given 70 or 21 pituitaries, the number of cancers was 8 of 10 and 5 of 5 respectively. Comparisons of these small samples of mice with different genetic background, receiving slightly different total numbers of treatments, do not reveal an effect of stock (and agent) or number of implants upon cancer incidence. Cancer age, in turn, seems to be affected by both factors, stock (agent) and number of implants. An analysis of variance (Table II) reveals these effects to be significant at about 10%. The difference in cancer age between pituitary isografted mice from the C and $D_8$ stocks is more clearly seen when the groups

TABLE II. Stock (Agent) and Number of Implants in Relation to Cancer Age.

| Stock | MTA | No. of implants | Cancer age (No. of mice) |
|---|---|---|---|
| C | — | 24 | 523 ± 32 (7) |
| | | 80 | 428 ± 40 (7) |
| $D_8$ | + | 21 | 411 ± 30 (5) |
| | | 70 | 391 ± 42 (8) |

Analysis of variance*

| Source | DF | MS | F† |
|---|---|---|---|
| No. of implants | 1 | .0373 | 3.08 |
| Stock (agent) | 1 | .0364 | 3.01 |
| Interaction | 1 | .0057 | <1.00 |
| Within | 23 | .0121 | |

* On $\log_{10}$ transformation of data.
† $F_{.90}(1, 23) = 2.94$.

receiving the smaller number of implants are compared [t computed for difference (in $\log_{10}$ transformed data) $= 2.56$; $P = .028$]. The effect of number of implants, in turn, appears more clearly in data on the C stock, not complicated by presence of MTA ($t = 1.868$; $P = .088$). In close agreement with Mühlbock and Boot(1,2), it seems that cancer age is shortened by either presence of MTA or by increase in number of implants; either effect, however, may be obscured by the other, that of the number of implants by the presence of the agent and vice versa. It also seems pertinent that in subsequent work from this laboratory, on 100 pituitary-isografted $D_8$ recipients, breast cancer incidence was over 20% by one year after implantation of only 2 pools, each composed of 3 pituitaries. For this stock, the number of implants is therefore not among the more critical experimental variables in mammary carcinogenesis from pituitary isografts.

*Effect of hypothalamus.* In some animals, a hypothalamus was inserted into the axilla (in the same trocar with the pituitary) at time of pituitary implantation. Cancer incidence in animals thus treated was slightly less than in mice given only the pituitary (Fig. 3) but the difference was not statistically significant in either stock. Noteworthy differences were found, however, in cancer age between animals given only the pituitary on one hand and the pituitary and hypothalamus on the other. At ~ 520 days post-implantation, one-half of the C mice given only pituitaries (5 out of 10) had breast cancer as compared to none of the (10) C mice given pituitary and hypothalamus ($P = .05$). The differences in cancer age as a function of whether or not the hypothalamus had been inserted were ultimately not significant at 10% in either stock. This lack of statistical significance in the C data, however, resulted from the fact that one animal from the group given only pituitaries developed cancer at a very late age and thus raised the mean cancer age of the pituitary group.

In the interim, additional groups each composed of 20 $D_8$ mice were given 2 pools of pituitary alone (I) or pituitary and muscle (II) or pituitary and cerebral cortex (III) or pituitary into one axilla and hypothalamus into the other axilla (IV), while another two groups (of 20 mice each) received the pituitary and hypothalamus in the same axilla either jointly by the same trocar (V) or successively through different trocars directed at the same site (VI). At the time of this writing, 11 months post-treatment, tumor incidence in the latter two groups (V and VI) is about one-fourth that in either of the former groups (I to IV). These latter findings confirm the impression gained from Fig. 3 and suggest further that mechanical factors cannot be held solely responsible for an apparent anti-cancer effect of the hypothalamus when it is inserted at the time of pituitary isografting. May has suggested that grafted dying tissues have chemical action while they are being replaced by immigrated elements and that nerve fibers may electively be attracted by tissues which are no longer, or which are not yet innervated(15). At any rate, the mode of action of the as yet ill-defined "hypothalamus"-insert (Fig. 1) awaits analysis with particular reference to the morphology or function of the grafted pituitary.

*Summary.* A light-dark synchronized activity rhythm persists in pituitary-isografted C mice. The adrenal cycle probably persists in mice with a significantly altered estrous cycle(1,2). Multiple pituitary isografts are associated with adenocarcinomata and adenoacanthomata in virgin female mice of the $D_8$ and C stocks. The carcinogenic effect of the pituitary isografts is statistically significant and not a result of trauma. Number of implants as well as presence or absence of mammary tumor agent also are significant variables, but neither variable is critical to carcinogenesis from pituitary isografts, under the conditions studied. Insertion of the hypothalamus at the time of pituitary implantation delays mammary carcinogenesis by mechanisms as yet unknown.

The authors are greatly indebted to Dr. Robert Hebbel of the Dept. of Pathology, Univ. of Minnesota, for valuable help in histologic diagnoses.

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# Apparent Loss of Glycine on Acid Hydrolysis. (25349)

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Studies concerning loss of amino acids during acid hydrolysis of protein containing materials have been reported(1,2). For the most part, such losses resulted from outright destruction, racemization, or irreversible binding to human material. The present report is concerned with an apparent loss of glycine, as determined microbiologically, with increasing time of acid hydrolysis. This loss is due to the presence of glycine peptides in the short time hydrolysates which promote greater growth of the assay organism than a corresponding amount of free glycine. The relationship of these findings to the antagonistic effects of certain amino acids in the basal medium upon glycine was investigated.

*Methods.* Hydrolysates were prepared by autoclaving 1 g of material with 20 ml of 3 N HCl in a sealed tube at 15 lb 121°C for varying time. The hydrolysates were taken to dryness, resuspended in water and made up to pH 4.0 with KOH. After filtering through a sintered glass funnel, the resulting clear filtrate was adjusted to pH 6.8, made to desired volume and stored under toluene at 5°C. Glycine was estimated microbiologically with *Leuconostoc mesenteroides* strain P-60 (ATCC 8042) using a commercially prepared amino acid assay mixture.* After distributing 2 ml of assay medium, samples and standards were added and total tube volume was made to 3 ml. The tubes were autoclaved for 10 min. at 15 lb 121°C, cooled, and inoculated with one drop of a washed suspension of test organism. Incubation was carried out at 37°C for 72 hours and the resulting acidity titrated with 0.025 N NaOH.

*Results.* Typical results illustrating effect of acid hydrolysis upon glycine content of soybean oil meal are shown in Fig. 1. The apparent glycine reaches a maximum at one hour, and thereafter drops rapidly to 6 hours. Additional autoclaving to 24 hours results in a further slight loss in activity. In separate experiments, where glycine was added prior to hydrolysis, recovery values ranging from 97.1 to 100.8% showed that this apparent loss was not due to destruction of free glycine. In addition to decrease in glycine activity with increased time of hydrolysis, a downward drift with increasing aliquot of sample assayed was observed. These results are shown in Table I. In the various hydrolysates, decrease in assayed glycine value is less with larger aliquot of sample and more pronounced with smaller aliquot. However, as time of hydrolysis is lengthened these differences become less significant.

Therefore, it seemed expedient to examine

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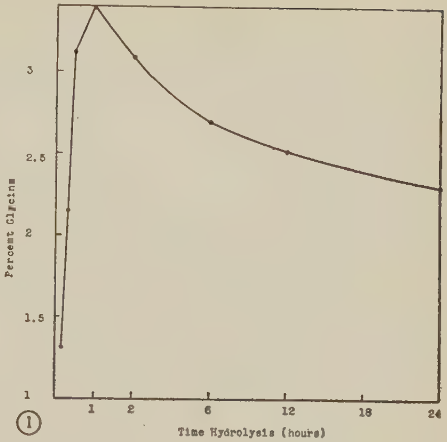
* H.M.Chemical Co.

TABLE I. Effect of Hydrolysis Time upon Glycine Content of Soybean Oil Meal.

| Hydrolysis time, hr | Sample wt, $\mu g$ | Glycine, % |
|---|---|---|
| 1 | 400 | 4.4 |
| | 800 | 3.7 |
| | 1200 | 3.3 |
| | 1600 | 3.0 |
| 2 | 400 | 4.0 |
| | 800 | 3.7 |
| | 1200 | 3.3 |
| | 1600 | 3.0 |
| 6 | 400 | 3.5 |
| | 800 | 3.3 |
| | 1200 | 3.0 |
| | 1600 | 2.8 |
| 12 | 400 | 2.8 |
| | 800 | 2.7 |
| | 1200 | 2.6 |
| | 1600 | 2.5 |

in more detail the nature of glycine activity present in both short- and long-term hydrolysates. According, 2- and 12-hour hydrolysates of soybean oil meal were passed through Dowex 50 columns, previously prepared in the hydrogen form, and eluted with successive volumes of water, 1.5, 2.5, and 4. N HCl. Several distinct fractions containing glycine activity were found in the 2-hour hydrolysate (Fig. 2). Of this total activity, approximately 20% was found in fractions which came off the column at points removed from that of free glycine. A quite different picture was obtained with the 12-hour hydrolysate (Fig. 3). All glycine activity was now found in one fraction which eluted from the column in a manner similar to that of free glycine. However, paper chromatograms and bioautographs of this fraction showed presence of glycine and an unidentified component which had glycine activity, gave an initially yellow ninhydrin reaction, and had an Rf value similar to that of glycylglycine.



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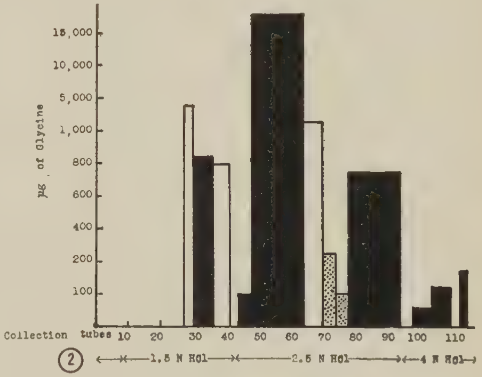

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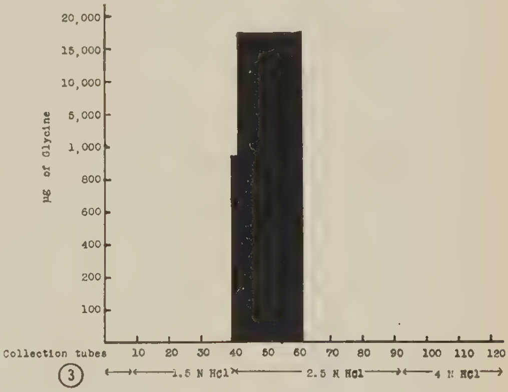

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FIG. 1. Effect of time of hydrolyses on glycine activity of soybean oil meal (44% protein).

FIG. 2. Passage of a 1-g, 2-hr soybean oil meal hydrolysate through a Dowex 50 column. Black bars were ninhydrin positive. Speckled bars gave a green ninhydrin. Blank bars were ninhydrin negative.

FIG. 3. Passage of a 1-g, 12-hr soybean meal hydrolysate through a Dowex 50 column.

*Discussion.* The most logical explanation of above results is that the 2-hour hydrolysate contains a variety of peptides which are largely hydrolyzed when the time is extended to 12 hours. It is also quite likely that the higher glycine values obtained with short-time hydrolysis as well as with smaller aliquots are due to presence of these peptides. In support of this supposition, it has been well established with *Leuconostoc mesenteroides* and other microorganisms that under certain conditions simple peptides may allow greater growth than their component amino acids(3). For example, with *L. mesenteroides,* growth at low levels of glycine is effectively inhibited by any one of several amino acids. However, the inhibitions may be overcome and growth restored by presenting glycine in the form of a simple dipeptide such as glycylglycine(4). Therefore, if the basal medium employed contained amino acids antagonistic to glycine at levels sufficiently high to inhibit growth, a greater growth promoting effect of glycine peptides as compared to free glycine would be expected. This was apparently the situation for when the alanine content of the medium was reduced to 1/20, leucine to 1/6, and the remaining amino acids by ½ of the original concentration, the short- and long-term hydrolysates, as well as various aliquots, gave equal growth (Table II). Therefore, in short-term hydrolysates, particularly, and somewhat even in the 12-hour hydrolysate, the peptide-bound glycine apparently allowed the assay organism to give greater growth in presence of antagonistic levels of various amino acids than an equivalent amount of free glycine. Comparison of such tubes to the standard glycine curve would necessarily give greater values. In the smaller aliquot, the effect of peptides is more striking than with the larger aliquot, because as quantity of glycine increases, inhibitory action of the antagonistic amino acids in the medium becomes less effective.

Although only data from experiments with soybean oil meal have been presented, similar results were obtained with casein, alfalfa, peanut meal, corn, wheat, blood meal, and fish meal. Using a medium modified as described above and a hydrolysis time of 2 hours, a number of feedstuffs have been assayed for glycine. The results of these assays are shown in Table III.

TABLE II. Effect of Media Change upon the Assay of Glycine in Soybean Oil Meal.

| | | % glycine | | |
|---|---|---|---|---|
| | | Time of hydrolysis | | |
| Media | Wt of sample, μg | 1 hr | 6 hr | 12 hr |
| Prepared | 400 | 4.4 | 3.5 | 2.8 |
| | 800 | 3.7 | 3.3 | 2.7 |
| | 1200 | 3.3 | 3.0 | 2.6 |
| | 1600 | 3.0 | 2.8 | 2.5 |
| Modified | 200 | 2.05 | 2.05 | 2.0 |
| | 400 | 2.0 | 2.02 | 1.97 |
| | 800 | 2.1 | 2.05 | 2.05 |

TABLE III. Glycine Content of Various Feedstuffs.*

| Feed | Protein, % | Glycine, % |
|---|---|---|
| Soybean oil meal | 43.3 | 2.1 |
| Alfalfa | 20.6 | 1.2 |
| Peanut meal | 44.2 | 2.4 |
| Corn | 8.7 | .4 |
| Wheat | 13.6 | .6 |
| Blood meal | 84.5 | 3.4 |
| Fish " | 57.3 | 4.2 |
| Casein | 80.0 | 1.8 |
| Cottonseed meal | 39.2 | 2.0 |
| Dried milk | 32.3 | .7 |
| Meat scraps | 52.9 | 7.5 |
| Oats | 12.3 | .6 |

* Air dried.

*Summary.* An apparent loss of glycine with increasing time of acid hydrolysis has been described. Short-term hydrolysates contain glycine peptides which largely disappeared as time of hydrolysis was extended. Lowering the concentration of amino acids in the basal medium resulted in disappearance of this apparent loss of glycine. With such a modified medium, a number of feedstuffs have been assayed for glycine.

Technical assistance of Melvin Whitehead is gratefully acknowledged.

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# A Melanophore-Stimulating Factor in Embryonic Skin. (25350)

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Preliminary experiments indicate presence of a factor in skin of *Ambystoma maculatum* tail-bud embryos capable of causing pigment dispersion in melanophores. Pigment-concentrating effect of hypophysectomy on amphibian melanophores has been known since the work of Smith(1). It has been reported, however(2-4), that extirpation of the epithelial anlage of the hypophysis is reflected by melanophores only after an initial period in which pigment cells appear morphologically identical to those in unoperated animals. In *A. maculatum, A. opacum* and *Siredon mexicanum* melanophores in hypophysectomized animals are maintained in stellate condition from their initial appearance, at Harrison stage 37, until embryos reach stage 41-42. A similar situation prevails in several anuran species(5), although they differ in length of time during which pigment cells remain stellate. Since for a period in development of the pigment pattern melanophores appear to be temporarily independent of pituitary for maintaining their stellate condition, we are faced with alternative hypotheses. Either (a) hormonal stimulation is unnecessary at first but required later for melanophores to maintain a dispersed arrangement of pigment granules, or (b) appropriate hormonal stimulation in tail-bud embryos comes from a source outside the pituitary gland. In either case the system operates only temporarily, between stage 37 and stage 41-42. The rudimentary condition of the circulatory system at these early stages suggested that, if a hormone were involved, its source might be tissues immediately adjacent to the integumentary pigment cells. The experiments reported here were undertaken to test the possibility that flank somatopleure in tail-bud embryos might contain temporarily a melanophore-stimulating principle, which might be the basis for the pituitary independence of melanophores in the initial pigment pattern of amphibian embryos.

*Materials and methods.* Flank ectoderm, with adhering hypomeric mesoderm cells, was stripped bilaterally from 46 *A. maculatum* embryos at stage 35. An homogenate was prepared from the pooled tissue in 1 ml of Holtfreter's Standard Solution. Two homogenates were prepared from 32 embryos of the black strain of *S. mexicanum* at stage 41-42, one from flank skin and one from tails of the same animals. Similar homogenates were also prepared from adult skin, heart, liver and brain of *Rana pipiens*. For testing the activity of homogenates, vesicles were prepared by removing from each *A. maculatum* embryo at stage 20 a rectangular piece of belly ectoderm. A piece of trunk neural crest from the same embryo was combined with the ectoderm, which rolls up to form a vesicle enclosing the neural crest tissue. As the vesicle, kept in Standard Solution, enlarges and becomes transparent, melanophores appear on the inner surface of the belly ectoderm fully punctate. In testing for melanophore-stimulating activity, vesicles and also hypophysectomized larvae were immersed in the crude homogenates and observed at 30-minute intervals. *Observations*: In the *A. maculatum* homogenate (stage 35 skin) melanophores in the vesicles and in hypophysectomized larvae began to disperse pigment after one hour and thirty minutes. The pigment cells appeared larger and a few cytoplasmic processes were visible. In the larvae this effect was restricted to the epidermal melanophores. At the end of three hours the vesicle melanophores had changed from their initial punctate condition to being stellate (from 1 on the Hogben scale to 3). After 12 hours pigment cells were graded 4 on the Hogben scale. Dermal melanophores in hypophysectomized larvae had dispersed pigment at the end of 3 hours, and after 12 hours both dermal and epidermal melanophores were at 3 on the Hogben scale. Homogenates prepared from *S. mexicanum* (stage 41-42 skin and tail) and from *R. pipi-*

*ens* (adult tissues) showed no pigment dispersing activity even after 12 hours.

*Discussion.* These results support the hypothesis that appropriate hormonal stimulation of initial melanophores comes temporarily from tissues outside the pituitary gland. The alternative hypothesis, that newly differentiated melanophores do not need hormones for maintaining dispersed pigment but later develop this need, is not excluded. It seems unlikely, however, that such an early autonomy is characteristic of melanophore differentiation because, if that were the case, hypophysectomized larvae should continue to show a small number of stellate melanophores representing the cells newly differentiated from melanoblasts in all parts of the pigment pattern(4) instead of appearing uniformly punctate over most of the body surface.

The evidence clearly indicates that something was extracted from embryonic flank skin at stage 35 which was capable of causing punctate melanophores to become stellate, both in epidermal vesicles and in hypophysectomized larvae. It might be thought that the active principle is really pituitary hormone which diffused through the embryo from the site of production in the hypophysis. While small but physiologically active amounts of MSH have been detected in pituitary anlagen even prior to cytological differentiation(5-8), in *A. maculatum* embryos Drager and Blount (8) were able to demonstrate pigment-dispersing activity in heads of embryos no earlier than stage 39. Furthermore, Kleinholz in *Rana pipiens* found no MSH activity in skin of embryos at any stage, even though activity was found in heads of some of the same embryos. Lastly, in our own experiments with homogenates of stage 41-42 embryonic skin, at an age when the pituitary may be supposed to be active, no pigment-dispersing activity was evident. The latter observation, together with the failure of homogenates of *R. pipiens* adult tissues to show melanophore-stimulating activity, is a check indicating that the active principle in the early embryonic skin is not an artifact released by the process of making the homogenates. Because of restrictions on availability of material, the sources of our embryonic homogenates differ in species as well as age. Preliminary interpretation from the results seems nonetheless justified because the patterns of melanophore development, including temporary pituitary independence, appear essentially the same in the species involved.

All these observations lead to the conclusion that there is a tissue-specific and time-specific occurrence of a melanophore-stimulating hormone in the embryonic skin of *A. maculatum* just at the period when the first melanophores appear in stellate condition in all embryos. At the age when melanophores in hypophysectomized embryos become punctate, melanophore-stimulating activity in embryonic skin is no longer demonstrable. How widespread among amphibian species this phenomenon may be must be further investigated. Kleinholz's report(6,7), that *R. pipiens* embryos of all ages show no pigment dispersing factor in skin, may not be definitive since in the process of testing he treated his embryonic samples with acetone prior to desiccation. Lee and Lerner(9) have shown that alpha-MSH is soluble in acetone. There is no evidence at this time concerning the chemical nature of the factor demonstrated in these experiments. Any supposition that early embryonic skin produces MSH, rather than acetyl choline or other humoral agent affecting melanophores, must remain speculative until further tests are made.

*Summary.* Homogenates from flank skin of *Ambystoma maculatum* embryos at stage 35 show MSH-like activity when tested with punctate melanophores in epidermal vesicles and in hypophysectomized larvae. Homogenates from flank skin of older embryos (*Siredon mexicanum* at stage 41-42) and from adult tissues (*Rana pipiens*) show no such activity. The temporary occurrence of an MSH-like factor in early embryonic skin may explain the initial pituitary independence of the earliest melanophores differentiating in urodele pigment patterns.

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## Further Characterization of Hemolytic Component of Fire Ant Venom, Mycological Aspects. (25351)

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Preliminary observations about the antibiotic character of venom were made by Caro *et al.*(3), who noted that of pustules produced by experimental stings, all, with one exception, were bacteriologically sterile. On one occasion non-hemolytic staphylococci were isolated, but organisms were not seen in tissue removed for biopsy. Using the paper disk method, Blum *et al.*(2) demonstrated the effectiveness of venom against *Micrococcus pyogenes, Streptococcus pyogenes, Escherichia coli, Lactobacillus casei* and a variety of molds which were not named. The present report is concerned with the antimycotic effects of crystalline, hemolytic component of fire ant venom isolated by Adrouny *et al.*(1). It is an attempt to add another means of biological characterization of the crystalline material and is not to be interpreted as a study on the antimycotic effects of this material for future clinical use.

TABLE I. Effect of the Crystalline Hemolytic Component of Fire Ant Venom on Fungal Growth.

| Inhibition of growth | No inhibition of growth |
|---|---|
| *Blastomyces dermatitidis* | *Epidermophyton floccosum* |
| *Candida albicans* | *Microsporum Audouini* |
| *Cladosporium Wernecki* | " *canis* |
| *Cryptococcus neoformans* | *Monosporium apiospermum* |
| *Geotrichum* sp. | *Oospora* sp. |
| *Hormodendrum compactum* | *Sporotrichum Schenckii* |
| *Hormodendrum Pedrosoi* | |
| *Microsporum gypseum* | |
| *Nocardia asteroides* | |
| " *brasiliensis* | |
| *Phialophora verrucosa* | |
| *Trichophyton mentagrophytes* | |
| *Trichophyton rubrum* | |
| " *tonsurans* | |
| *Trichosporon Beigelii* | |

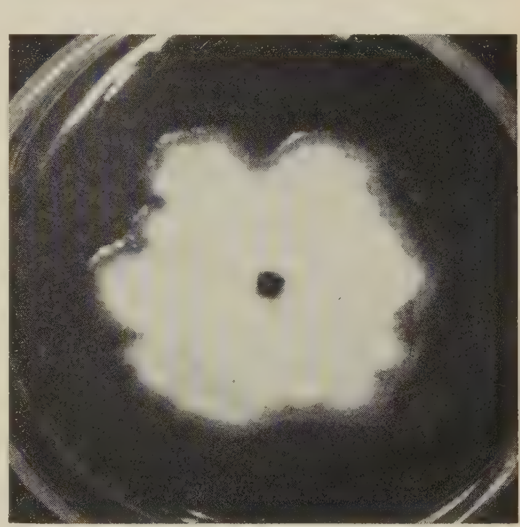

FIG. 1. Inhibition of *Trichophyton rubrum* by a crystal of hemolytic component of fire ant venom.

*Methods.* A series of fungi, mostly human pathogens, were streaked onto Petri dishes containing a medium consisting of 2% glucose, 1% yeast extract, 1% casamino acids and 2% agar in distilled water. All cultures were tested with a single lot of crude crystalline venom which had a melting point of 139-140°C and was insoluble in aqueous media. A few cultures were also tested with a recrystallized venom having a melting point of 144°-

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* Post-doctoral research trainee in medical mycology. Investigation supported by research training grant, and research grant from P.H.S.

145°C similarly insoluble. A small crystal of venom was placed on each of the seeded plates which were then incubated at room temperature (25°-30°C). All crystals were approximately but not exactly the same size. The results were recorded when growth was at its maximum.

*Results.* Of 21 organisms tested, 15 were inhibited by the hemolytic component of fire ant venom. (Table I). Results obtained with the 2 lots of crystals were similar. An example is shown in Fig. 1.

*Summary.* The crystalline hemolytic component of fire ant venom was tested against 21 organisms, mostly human pathogens, by placing a crystal in direct contact with Petri dishes seeded with a different organism. Antimycotic action was demonstrated by a lack of growth immediately surrounding the crystal with the following genera: *Blastomyces, Candida, Cladosporium, Cryptococcus, Geotrichum, Hormodendrum, Microsporum, Nocardia, Phialophora,* and *Trichophyton.*

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## Antioxidant Effect of Chlorpromazine. (25352)

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Phenothiazine and its derivatives have been known for many years as potent antioxidants, and are used in the petroleum industry for that purpose(1). This fact suggested their trial as inhibitors of formation of lipide peroxides in biological systems. Rat brain and liver homogenates and liver mitochondria were used, and the amount of fatty acid peroxide was estimated by the thiobarbituric acid method(2). Chlorpromazine, and derivatives which are formed from it on standing in light, markedly inhibit formation of peroxides, and some properties of the reaction are described below.

*Methods.* Rat liver or brain was removed, iced, homogenized in 0.05 M phosphate buffer at pH 6.8, and diluted 1:10. Usually 0.4 ml of the homogenate was added to the drug solution and water to make 2 ml, and the flasks incubated aerobically at 38°. After incubation, 4 ml 20% trichloracetic acid were added, then 3 ml water, and 1 ml 0.75% thiobarbituric acid solution. The mixture was heated for 15 min in boiling water bath, centrifuged, and the red color produced by the reaction of fatty acid peroxides with thiobarbituric acid was estimated using a 520 filter. Color units are arbitrary.

*Results.* When solutions of chlorpromazine remained in light they rapidly darkened. These colored solutions gave no absorption peaks in the visible spectrum. The chemistry of change is not fully understood and quantitative relationship of the dark colored product to the original compound is therefore unknown. However, with the homogenized brain or liver, the antioxidant activity of the colored solution was about 5 times that of fresh material. Maximal activation occurred in a few days, and recognizable activation could also be observed in a solution kept for 24 hours at 38° in the dark. Fig. 1 shows that lipide peroxide formation in liver homogenates by both original and "activated" chlorpromazine was proportional, within limits, to their concentrations. If the concentration of chlorpromazine was kept constant and amount of liver homogenate varied, there was a linear decrease of inhibition with increasing amounts of homogenate. Thus, with 0.8, 0.4 and 0.2 ml of homogenate, 12.5 $\mu$g/ml chlorpromazine inhibited 21, 46, and 90% respectively. Percent inhibition by both chlorpromazine and its "activated" product was constant throughout the incubation period, when measured at intervals between 15 and 90 min. Thus the liver

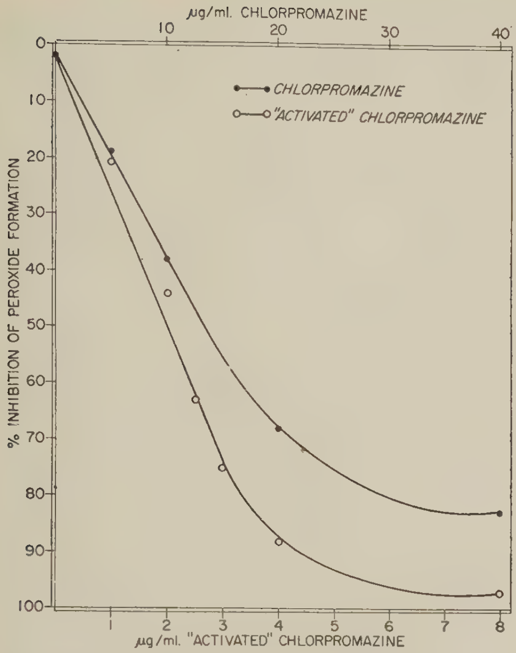

FIG. 1. Inhibition by chlorpromazine of lipide peroxide formation in rat liver homogenate incubated 60 min. at 38°; pH 6.8. "Activated" chlorpromazine was the material in solution for 4 days in the light at room temperature before use.

did not alter antioxidant activity of either compound.

Sulfoxide of chlorpromazine was 10 times less effective an inhibitor than chlorpromazine on both brain and liver. However, it too darkened on standing, and became, like chlorpromazine, 5 times more active than the original solution. Methylene Blue also inhibited production of fatty acid peroxides and on a molar basis was as active as the "activated" chlorpromazine.

When ferrous ions are added to a mitochondrial suspension, formation of lipide peroxide is much increased. A concentration of 1 x $10^{-3}$ M $Fe^{++}$ increased the thiobarbituric acid color value after 1 hour incubation from 15 to 300, and this effect can be prevented if EDTA or other chelating agents are added to the system(3). Chlorpromazine, however, inhibited formation of color in very much smaller concentrations, *i.e.*, 5 x $10^{-5}$ M, so that chelation of only a small part of the $Fe^{++}$ could have taken place. The chlorpromazine effect is therefore the result of some other process.

Addition of ferric iron to fresh chlorpromazine in acid causes rapid formation of a pink color, which shows maximum absorption at 530 mm and 255 mm, and fades within a few hours(4). This product was tested on liver homogenates, but was not more active than chlorpromazine.

*Discussion.* Although the antioxidant activity of chlorpromazine and its "activated" product is demonstrable *in vitro* in concentrations which might be present during clinical use of the drug, it is improbable that the overall pharmacological actions can be explained on this basis. However, lipides are known to be concerned with electron transport systems, and *in vitro* experiments have shown that chlorpromazine inhibits oxidation and oxidative phosphorylation in mitochondria(5). Antioxidant activity of chlorpromazine may be correlated with its ability to inhibit mitochondrial systems, in which case the "activated" form would be expected to be more effective than the fresh drug.

It does not seem likely that the antioxidant effect is related to chelation of metals; more probably, the action is connected with the tendency of phenothiazine and its derivatives to form resonance-stabilized free radicals. Their ability to inhibit lipide peroxide formation in brain and liver homogenates decreases in the following order:—Methylene Blue = "activated" chlorpromazine > chlorpromazine > "activated" chlorpromazine sulfoxide > chlorpromazine sulfoxide.

*Summary.* 1. Chlorpromazine, in concentrations of 2 x $10^{-5}$ M, inhibits formation of lipide peroxides in liver and brain homogenates. 2. When allowed to remain in light, chlorpromazine solutions become brown in color, and the activity is increased 5-fold. 3. Chlorpromazine sulfoxide is less effective than chlorpromazine, but is similarly activated on standing. 4. Methylene blue is a powerful antioxidant, comparable to the activated form of chlorpromazine.

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# Digestion, Absorption and Metabolism of Chimyl Alcohol Fed as Free Alcohol or as Alkoxydiglyceride.* (25353)

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It was shown previously(1,2) that after feeding free chimyl alcohol dissolved in olive oil to rats or to human beings, the ether linkage was broken in the intestinal mucosa so that about 50% of radioactivity in lymph was associated with palmitic acid and the remainder of chimyl alcohol was present partly as chimyl alcohol and partly as esterified. This finding together with structural similarity of chimyl alcohol with a monoglyceride makes it possible to study whether a real synthesis of glycerides takes place in the intestinal contents and the forms in which dietary triglycerides are absorbed into the intestinal mucosa. In the present report the course of hydrolysis and synthesis of alkoxydiglycerides *in vivo* was followed after feeding labelled chimyl dioleate or free chimyl alcohol to rats. Furthermore metabolism and distribution of chimyl alcohol in the rat has been followed.

*Methods.* Labelled material: Preparation of $\alpha$-(1-$C^{14}$)-hexadecylglyceryl ether (chimyl alcohol) was carried out mainly according to Holmes *et al.*(3) as described earlier(1,2). Radioactive chimyl dioleate was prepared by esterification of $\alpha$-(1-$C^{14}$)-hexadecylglyceryl ether with oleic acid chloride in pyridine. Acid chloride was prepared from oxalyl chloride. Free fatty acids were removed by passing the mixture through Amberlite IRA-400. The neutral material was then applied in benzene to a silicic acid column and eluted with successive portions of benzene, benzene:chloroform (85:15) and chloroform. More than 95% of radioactive material was eluted with benzene. The diesterified chimyl alcohol (chimyl dioleate) thus behaved as a triglyceride. This chromatographic behavior of alkoxydiglyceride has been described(1,2). Labelled chimyl alcohol was fed as free alcohol or as chimyl dioleate dissolved in olive oil. In one series (A) intestinal contents were analyzed during absorption phase 2-4 hours after administration. In another series (B) distribution of activity in liver lipides and expired $CO_2$ was determined 24 hours after administration. Finally in some experiments (C) distribution of activity in lymph lipids was followed after feeding chimyl dioleate. A. Rats were fed 0.5 ml of 2% solution of chimyl dioleate or chimyl alcohol in olive oil by stomach tube. Animals were killed 2-4 hours after administration, and contents of small intestine were washed out with saline into dilute hydrochloric acid. Total fat was extracted 3 times with 2 volumes of ether. Separation of free fatty acids and glycerides of recovered lipides was performed on columns of Amberlite-IRA-400. A further separation of glycerides into tri-, di-, and monoglycerides was made on columns of silicic acid(4). In this separation chimyl dioleate followed the triglycerides, chimyl monooleate the diglycerides, and chimyl alcohol the monoglyceride fraction. B. In some experiments the same solutions as in Exp. A were fed to rats, expired $CO_2$ was collected and isotope content determined as described earlier(5). For comparison chimyl alcohol was injected intravenously and expired $CO_2$ collected. At different time intervals 24-72 hours, the rats were sacrificed and the organs analyzed. In the present paper only data on excretion of radioactivity in the breath are given. C. In a few experiments, labelled chimyl dioleate dissolved in olive oil was fed to rats with a thoracic duct fistula. The technic for collection of lymph

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TABLE I. Composition and Isotope Content in Lipides Recovered from Intestinal Washings after Feeding Free $C^{14}$-Labelled Chimyl Alcohol Dissolved in Olive Oil to 2 Rats. Animals were killed after 2 and 4 hr respectively. Exp. A and B. Free chimyl alcohol followed the monoglycerides in separation on silicic acid. Specific activity of fed mixture 569 cpm/mg.

| Chromatographic fractions | % of total lipides | | Specific activity, cpm/mg | | % of total $C^{14}$-activity | |
|---|---|---|---|---|---|---|
| | A | B | A | B | A | B |
| Triglycerides | 23.6 | 16.5 | 147 | 213 | 14.4 | 13.6 |
| Diglycerides | 23.6 | 13.2 | 240 | 836 | 16.3 | 31.0 |
| Monoglycerides | 22.4 | 20.0 | 1060 | 870 | 68.8 | 51.7 |
| Free fatty acids | 30.4 | 50.3 | 6 | 27 | .5 | 3.7 |

and the analyses of lymph has been described (1).

*Results.* Composition of lipides recovered from contents of small intestine of rats fed free chimyl alcohol or chimyl dioleate dissolved in olive oil is summarized in Tables I and II. Although there are great variations, values for the different components agree well with those of Desnuelle and Constantine(6), Mattson *et al.*(7), and Borgström(8). Monoglyceride values represent total monoglycerides including free chimyl alcohol. Chimyl alcohol with one fatty acid attached is included in the diglyceride fraction and chimyl alcohol with 2 fatty acids attached follows the triglyceride fraction in separation on silicic acid.

In Table I results of activity determinations of different mono-, di-, and triglyceride fractions and of free fatty acids are summarized. No significant activity was associated with free fatty acids and none was found in the fatty acids of different glyceride fractions, indicating that radioactivity in the different glycerides is due to the chimyl alcohol component of these fractions.

When radioactivity of the glyceride fractions is considered (Table I), it appears that the monoglyceride fraction has the highest specific activity and the triglyceride fraction the lowest. However, the radioactivity associated with the di-, and triglyceride fractions is significant, and in Exp. B specific activity of the diglyceride fraction is even higher than that of the fed mixture, indicating a synthesis of glyceride ester bonds taking place simultaneously with hydrolysis.

Experiments in which chimyl dioleate dissolved in corn oil was fed are summarized in Table II. After feeding this mixture significant amounts of label were obtained in mono-. di-, and triglycerides. Because no significant activity was associated with the free fatty acids, no synthesis of glycerides from labelled fatty acids could have taken place. Of the glycerides the diglyceride fraction had the highest and the triglyceride fraction the lowest specific activity. The monoglyceride fraction, which includes free chimyl alcohol, had a relative specific activity of 158%, indicating a concentration of free chimyl alcohol, in which both original fatty acids had been released by enzymatic action. The results thus indicate that fatty acids in 1- and 2-position of the alkoxydiglyceride had been attacked by enzymes present in the intestinal content.

The results of some lymph experiments are given in Table III. About 75% of radioactivity in the lymph was present as glyceride fatty acids and about 2% as phospholipid fatty acids. About 25% of lymph activity was present as chimyl alcohol of which more

TABLE II. Summary of Analyses of Intestinal Contents of 2 Rats after Feeding $C^{14}$-Labelled Chimyl Alcohol Esterified with Oleic Acid (Chimyl Dioleate) and Dissolved in Olive Oil. Rats were sacrificed after 4 hr, and total fat separated into tri-, di-, and monoglycerides on silicic acid. In this separation chimyl dioleate followed the triglycerides and free chimyl alcohol the monoglyceride fraction. Specific activity of fed mixture 1747 cpm/mg.

| Chromatographic fractions | Total wt of resp. fractions, mg | % of total fat | Spec. act., cpm/mg | % of total activity |
|---|---|---|---|---|
| Triglycerides | 27.3 | 24.7 | 1748 | 33.0 |
| Diglycerides | 13.0 | 11.6 | 3571 | 32.1 |
| Monoglycerides | 18.0 | 16.4 | 2743 | 34.0 |
| Free fatty acids | 52.7 | 47.3 | 27 | .9 |

TABLE III. Recovery and Distribution of Radioactivity in Lymph Lipids after Feeding $C^{14}$-Labelled Chimyl Dioleate Dissolved in Olive Oil to 2 Rats with a Thoracic Duct Fistula.

| % of admin. activity absorbed | % of absorbed activity recovered in lymph lipids | % of activity in lymph lipids recovered as: Chimyl alcohol Free | Chimyl alcohol Esterified | Triglyceride fatty acids | Phospholipid fatty acids |
|---|---|---|---|---|---|
| 90 | 60 | 6 | 18 | 74 | 2 |
| 85 | 45 | 5 | 20 | 73 | 2 |

than two-thirds was present as esterified chimyl alcohol. These results are similar to those obtained earlier(1,2) after feeding free chimyl alcohol to rats.

Appearance of $C^{14}$ in expired $CO_2$ is shown in Table IV. A significant difference in rate of metabolism of chimyl alcohol and palmitic acid is demonstrated. Chimyl alcohol is metabolized to $CO_2$ only half as fast as palmitic acid. After intravenous injection of chimyl alcohol, there is a rapid appearance of labelled $CO_2$ demonstrating widespread occurrence of enzymes capable of splitting the ether bond.

*Discussion.* Our results with free chimyl alcohol (Table I) clearly show that incorporation of free fatty acids into chimyl alcohol with formation of alkoxydiglycerides is due to synthesis of new glyceride-ester bonds. These experiments thus with a different approach, support earlier findings of Borgström(7) that a resynthesis of glyceride ester bonds occurs simultaneously with the hydrolysis in the lumen of small intestine. Our results therefore give no support to the statement of Reiser and Dieckert(9) that no significant amount of fatty acid exchange occurs in the intestinal lumen of rat during fat digestion.

The results of analyses of intestinal contents together with lymph analyses of this and previous investigations(1,2) clearly indicate the dynamic situation in the intestine. In the intestinal lumen, ether linkage is intact during the whole absorption process, but once absorbed into the intestinal mucosa more than 50% of the compound is rapidly converted into palmitic acid, indicating a splitting of the ether bond.

TABLE IV. Excretion of Radioactivity in Breath by Rats after Feeding: A. Palmitic acid-1-$C^{14}$ dissolved in olive oil, B. $C^{14}$-labelled chimyl alcohol esterified with oleic acid (chimyl dioleate) dissolved in olive oil, and C. Intrav. inj. of free $C^{14}$-labelled chimyl alcohol. Figures are the means of 2 exp.

| Exp. | Hr after dose | Cumulative % of total radioactivity admin., excreted as $C^{14}O_2$ |
|---|---|---|
| A | 24 | 16 |
| B | 24 | 8 |
| C | 24 | 24 |

Our results (Table III) indicate that fatty acids in both 1- and 2-position in the alkoxydiglyceride molecule are exchanged during hydrolysis by pancreatic enzymes *in vivo*. Apparently an equilibrium is soon reached between synthesis and hydrolysis of mono- and di-esterified chimyl alcohol and free chimyl alcohol.

Earlier resynthesis of glyceride ester bonds during hydrolysis of glycerides has been evidenced for the step 1,2-diglyceride to triglyceride and a synthesis from a 2-monoglyceride has appeared likely(8,10). The present results clearly indicate that a synthesis from a 1-monoglyceride is possible, because significant amounts of radioactivity were associated with the triglyceride fraction after feeding free chimyl alcohol.

Our results must be considered as proof that glycerol monoethers can be absorbed unchanged. However, the results also indicate that chimyl alcohol is absorbed with one or 2 fatty acids attached. This finding indicates that dietary glycerides are absorbed as a mixture of mono-, di-, and triglycerides and free fatty acids(11). The important role of the intestinal mucosa in formation of final products which are fed into the intestinal lymph is emphasized in our investigation.

*Summary.* 1. $C^{14}$-labelled chimyl alcohol ($\alpha$-/1-$C^{14}$/-hexadecylglyceryl ether) has been fed as free chimyl alcohol or as chimyl dioleate to rats. Intestinal contents and thoracic duct lymph lipids have been analyzed and expired $CO_2$ has been collected. 2. Mixed ether-glycerides have been isolated from the intestinal contents after feeding free chimyl alcohol dissolved in olive oil, indicating a synthe-

sis of new glyceride ester bonds. 3. Free labelled chimyl alcohol has been isolated from the intestinal contents after feeding labelled chimyl dioleate indicating an exchange and release of fatty acids in both 1-, and 2-position in the mixed ether-glyceride. 4. In lymph lipids more than half the radioactivity was associated with palmitic acid, indicating a splitting of the ether bond in mucosa cells. 5. It is concluded that chimyl alcohol can be absorbed unchanged but that it is extensively metabolized already in the mucosa cells, whether it is fed as free chimyl alcohol or as an alkoxydiglyceride.

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# Inhibition of Gastric Secretion in the Rat by Normal Human Gastric Juice.*

(25354)

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Presence of a substance in normal human gastric juice which inhibits Heidenhain pouch secretion in dogs when administered intravenously has now been well established(1,2). This substance is not dialyzable, is resistant to boiling and is largely inactivated by drastic acidification(2). It appears to inhibit specifically hydrochloric acid and water secretion (2). Studies aimed at identification and isolation of the active substance have been hindered by certain disadvantages inherent to the Heidenhain pouch preparation. Of these, the foremost is gradual development of gastric atrophy after the animal received several injections of gastric juice(3). Our purpose was to determine the feasibility of using the pylorus ligated rat preparation(4) for determination of inhibitory activity of preparations of normal human gastric juice.

*Materials and methods.* Gastric juice was collected from hospital patients previously found free of any gastrointestinal pathology. Following an overnight fast, a 4 hour collection of insulin stimulated gastric secretion was carried out. Immediately after collection, the juice from several subjects was pooled, dialyzed 36 hours against distilled water and lyophilized. Approximately 1 mg of material/1 ml of gastric juice was obtained. Immediately prior to use, this material was resuspended in normal saline and brought to pH 7.0. Gastric secretion was measured by pylorus ligation technic(4) in 65 male Holtzman rats weighing 160 to 220 g. Prior to experiments, rats were fasted 48 hours in individual cages with wide mesh wire bottoms. Water was given *ad lib*. Rats were operated upon under light ether anesthesia, and pylorus was ligated with a 4-0 silk ligature. Rats were divided randomly into control group and 3 treatment groups. Control rats were given 1 ml of normal saline; treated rats received

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TABLE I. Effects of Human Serum Albumin and Normal Human Gastric Juice on 4 Hour Gastric Secretion in the Pylorus Ligated Rat.

| | No. rats | Wt (g)† | Vol (ml)† | | Free HCl (meq/l)† | |
|---|---|---|---|---|---|---|
| Controls, 1 ml saline* | 17 | 181 ± 12 | 6.3 ± 1.5 | | 110 ± 22 | |
| Treated, 6 mg albumin* | 15 | 179 ± 10 | 6.0 ± 1.7 | | 111 ± 7 | |
| ” , 2 mg human gastric juice* | 14 | 176 ± 12 | 3.2 ± 1.8 | p .001 | 94 ± 25 | p .1 |
| ” , 6 mg ” ” ” * | 19 | 175 ± 14 | 1.3 ± .6 | p .001 | 43 ± 28 | p .001 |

* Intrav. inj. † Mean ± stand. dev. p = probability of significance of t value.

either 6 mg of human serum albumin, 6 mg of normal human gastric juice or 2 mg of normal human gastric juice in 1 ml of normal saline. Doses were on basis of 200 g of rat weight and were given intravenously by single injection into the vena cava at time of pyloric ligation. Exactly 4 hours later the animals were killed by exsanguination, the stomachs removed and gastric content was measured individually for volume, free acidity and in some instances for peptic activity. The latter was measured by modification of the method of Anson and Mirsky(5).

*Results.* The results are summarized in the Table. In animals receiving normal saline the mean volume of 4 hour gastric secretion was 6.3 ml with a free HCl concentration of 110 meq/l. Essentially no changes in these values occurred in rats given human serum albumin. In rats given 2 mg of human gastric juice the mean volume of 4 hour gastric secretion was 3.2 ml with a free HCl concentration of 94 meq/l. These values were 1.3 ml and 43 meq/l respectively in rats receiving 6 mg of the material. In the few experiments in which it was measured, peptic activity of gastric content was not altered by administration of human gastric juice.

Rectal temperatures of rats were taken before and after injections. No fever was produced by the gastric juice preparation.

*Discussion.* Characteristically, the rat stomach secretes a large volume of highly acid interdigestive secretion which is quite consistent from one animal to another of similar weight. For this reason the rat is an excellent animal in which to study agencies inhibiting gastric secretion. Previous attempts to use the rat for assay of other biologic gastric inhibitors such as enterogastrone(6) have failed. However, it appears that "gastrone" which is the name given by Code to the inhibitory substance present in human gastric juice has a consistent inhibitory effect on the stomach of the rat. Although doses required to produce inhibition in rats are relatively larger than those resulting in the same degree of inhibition in dogs, the total doses are considerably smaller.

No change in pepsin output was observed, thus confirming an earlier observation on dogs (2); the inhibitory effect is specific for water and acid secretion. Failure of human serum albumin to alter gastric secretion suggests that the action of human gastric juice is not a nonspecific one due to a foreign protein.

The active principle in human gastric juice responsible for inhibition of gastric secretion in animals has not yet been identified. The recently described resin column chromatography of Richmond *et al.*(7) for separation of large molecular components of gastric juice may be useful in isolation of the inhibitory factor. Considering the small amounts of fractionated material available for assay and the many assays which may be necessary, the pylorus ligated rat preparation appears to be the most satisfactory test preparation for this purpose.

*Summary.* Human gastric juice from subjects free of gastrointestinal pathology was collected, pooled, dialyzed and lyophilized. The material thus obtained consistently inhibited gastric secretion of pylorus ligated rats when administered intravenously in amounts of 2 or 6 mg at time of pyloric ligation.

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# Counting Efficiency of $Co^{56}$, $Co^{58}$ and $Co^{60}$ with 2 Counters and 3 Counting Technics. (25355)

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The technic for measuring Vit. $B_{12}Co^{60}$ of low specific activity contained in one liter of solution on a NaI well scintillation counter has been described(1). Hine and Miller(2) reported on efficiency of counting Vit. $B_{12}Co^{60}$ in a large plastic scintillator capable of holding 60-80 ml. Recently the $Co^{58}$ and $Co^{56}$ isotopes have been used in studies with radioactive Vit. $B_{12}$. This paper reports on the counting efficiency of $Co^{56}$, $Co^{58}$, $Co^{60}$ as a function of sample volume in both a modified plastic scintillator and the conventional NaI well and the merit of the counting systems

*Methods.* Vit. $B_{12}Co^{58}$ and Vit. $B_{12}Co^{60}$ were purchased from Merck & Co. and Vit. $B_{12}Co^{56}$ was generously supplied by Dr. E. Lester Smith of Glaxo Labs., Greenford, England. 0.25 $\mu$c aliquots of Vit. $B_{12}Co^{56}$, $Co^{58}$ and $Co^{60}$ were counted in the NaI well using a standard vial up to a maximum capacity of 4 ml and in a beaker containing one liter adapted to fit on and around the NaI well. In addition the 3 isotopes were counted in the plastic well scintillation counter. The counting efficiency of the 3 isotopes as a function of sample volume was compared. The NaI well type scintillation counter for counting up to 4 ml samples as well as the beaker to fit on and around the counter capable of counting 1 liter of solution was previously described(1). The counter* consists of an RCA 5819 phototube, a thallium activated sodium iodide crystal, 1¾″ diameter and 1″ thick with a hole ¾″ in diameter and 1″ deep, and a cathode follower preamplifier. The plastic scintillator† was modified and enlarged on the advice of Dr. G. J. Hine from that previously described and diagram shown in Fig. 1. The plastic well was surrounded by 3 in. of lead shielding except at the tube opening. Test tubes to contain the samples in this well were 38 x 300 mm.

*Results. Small Volumes (1-4 ml.)* If one counts samples in small volumes (1-4 ml), the capacity of most commercially available NaI well crystals, it is apparent from Table I that the plastic well is as efficient as the NaI well for $Co^{56}$ and more efficient than the NaI well for $Co^{58}$ and $Co^{60}$. The NaI crystal used in these studies is slightly less efficient than the more recent commercially available crystals. Since average background rate of the plastic well is about 400 cpm as compared to 120 cpm for the NaI crystal, the ratio of $R_S^2/R_b$ ($R_S$ = total counting rate, $R_b$ = background rate) is greater for the NaI well. It has been shown that the higher the ratio of $R_S^2/R_b$ (merit ratio) the less the counting time necessary for a given accuracy(3).

*Intermediate Volumes (4 ml-250 ml).* The plastic scintillator is highly efficient and the efficiency is almost independent of sample size from 10-100 ml for the 3 isotopes. Larger NaI wells holding more than 4 ml are difficult and very expensive to build. Baskin *et al.*(4) described a NaI well crystal with a capacity of 20 ml and an efficiency for $Co^{60}$ of 22.6%. Thus the plastic well appears to be the most efficient method for counting gamma radiation contained in sample volumes of 10 to 250 ml. Because of relative independence of volume size on efficiency and the larger size of plastic well, whole mice, rats up to about 75 g and

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* Model $S_1$—purchased from H. O. Anger, Berkeley, Calif.

† Pilot Scintillator B, Pilot Chemicals. Specific gravity = 1.03 Atomic ratio $\frac{H}{C}$ = 1.10

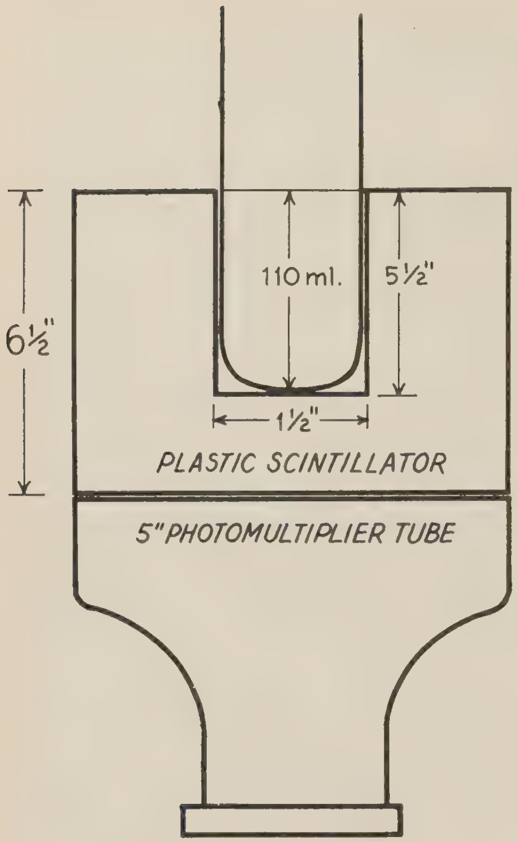

FIG. 1. Plastic scintillator

tissue samples can conveniently be counted in the plastic scintillator without corrections for geometry.

*Large Volumes (Above 250 ml)* Efficiency of about 200 ml in the plastic well is approximately 5 times the efficiency of 1000 ml using beaker and NaI well with the $Co^{58}$ isotope. Thus if 1000 ml samples are available, such as 24 hour urine collections, 200 ml may be counted in the plastic well or 1000 ml in the beaker with the NaI well with equal sensitivity. The choice of instrument to use in this instance would depend upon number of samples to be counted and activity of samples. The higher background rate of the plastic well would require longer counting time, but the relatively more expensive specially designed beaker for the NaI well as compared to the general availability of test tubes for the plastic well would require that the beaker be washed before another sample could be counted. If more than 1000 ml samples are available, the beaker would be the most sensitive technic for counting. For example, if a 2000 ml sample were available, one could count half of this sample in the beaker with the NaI well at 3.5% efficiency for $Co^{58}$ as compared to 1/10 the sample at 16.5% efficiency. If on the other hand, sample volumes of 100 ml to 1000 ml were available, the instrument of greatest sensitivity is the plastic well. With $Co^{56}$, the plastic well with a 200 ml sample is only about 3.5 times the efficiency (28.0% *vs.* 7.8%) of 1000 ml with the beaker and NaI well. In this instance it would be more advantageous to use the beaker with sample volumes of 700 ml or greater. With $Co^{60}$ the efficiency of the plastic well at 200 ml is 6 times the efficiency (22.6% *vs.* 3.8%) of 1000 ml with the beaker and NaI well and it would not be any more advantageous to use the beaker unless the sample size was 1200 ml or greater.

*Comparison of Beaker with NaI well* vs. *NaI well alone.* As pointed out(1) 1000 ml samples of $Co^{60}$ in the beaker over the NaI well results in an efficiency of about 1/6 (3.8% *vs.* 23.2%) that obtained by counting the same amount of radioactivity in a 4 ml volume using a vial placed directly in the well. However, in using a 1000 ml sample, one is counting 250 times more sample material. Multiplying 250 by 1/6 it is apparent that the beaker is more than 40 times more sensitive than the NaI well using just the vial. This is equally true with the $Co^{56}$ and $Co^{58}$ isotopes. The efficiency of $Co^{56}$ and $Co^{58}$ in 4 ml in the vial is 46.8% and 20.5% respectively. The efficiency of $Co^{56}$ and $Co^{58}$ in a 1000 ml sample in the beaker is 7.8% and 3.5% respectively.

*Summary.* A NaI well scintillation counter and a beaker adapted to fit on and around the NaI well as well as a plastic well scintillation counter, were used to compare efficiency of $Co^{56}$, $Co^{58}$, and $Co^{60}$ as a function of volume. The plastic well counter is as efficient for $Co^{56}$ as the NaI well and more efficient for $Co^{58}$ and $Co^{60}$ than the NaI well for small volumes (1-4 ml). For intermediate volumes (4-250 ml) the plastic well is the more efficient and the efficiency is almost independent of sample

TABLE I. Efficiency and Ratio of $R_s^2/R_b$ of $Co^{56}$, $Co^{58}$ and $Co^{60}$ in NaI Well Scintillation Counter and in Large Plastic Scintillation Counter as a Function of Volume.

| | $Co^{56}$ | | $Co^{58}$ | | $Co^{60}$ | |
|---|---|---|---|---|---|---|
| NaI well | Efficiency (%) | $R_s^2/R_b$ | Efficiency (%) | $R_s^2/R_b$ | Efficiency (%) | $R_s^2/R_b$ |
| Vial 1 ml | 59.6 | $14.5 \times 10^9$ | 25.7 | $2.7 \times 10^9$ | 26.3 | $2.8 \times 10^9$ |
| 2 | 56.8 | 13.4 " | 24.0 | 2.4 " | 27.3 | 3.1 " |
| 4 | 46.8 | 8.9 " | 20.5 | 1.7 " | 23.2 | 2.2 " |
| Beaker 100 | 7.2 | $20.8 \times 10^7$ | 3.1 | $3.7 \times 10^7$ | 3.4 | $4.7 \times 10^7$ |
| 200 | 7.3 | 21.8 " | 3.1 | 4.0 " | 3.6 | 5.2 " |
| 400 | 8.2 | 27.6 " | 3.6 | 5.3 " | 4.0 | 6.7 " |
| 600 | 8.7 | 30.4 " | 3.8 | 5.9 " | 4.2 | 7.3 " |
| 800 | 8.5 | 29.1 " | 3.7 | 5.6 " | 4.1 | 7.0 " |
| 1000 | 7.5 | 24.4 " | 3.5 | 4.9 " | 3.8 | 6.0 " |
| Plastic well | | | | | | |
| 1 | 52.6 | $3.4 \times 10^9$ | 31.6 | $1.2 \times 10^9$ | 39.4 | $1.9 \times 10^9$ |
| 2 | 56.6 | 4.0 " | 30.3 | 1.1 " | 39.3 | 1.9 " |
| 4 | 49.6 | 3.1 " | 28.6 | 1.0 " | 37.9 | 1.8 " |
| 10 | 47.6 | 2.8 " | 28.5 | 1.0 " | 39.2 | 1.9 " |
| 20 | 47.4 | 2.7 " | 28.1 | 1.0 " | 38.8 | 1.9 " |
| 40 | 46.2 | 2.6 " | 28.4 | 1.0 " | 38.3 | 1.8 " |
| 60 | 45.8 | 2.6 " | 27.7 | .9 " | 37.2 | 1.7 " |
| 80 | 45.0 | 2.5 " | 27.5 | .9 " | 36.8 | 1.7 " |
| 100 | 43.4 | 2.3 " | 26.0 | .8 " | 35.8 | 1.6 " |
| 125 | 40.0 | 1.9 " | 24.1 | .7 " | 32.8 | 1.3 " |
| 150 | 35.0 | 1.5 " | 21.2 | .6 " | 29.2 | 1.1 " |
| 175 | 31.4 | 1.2 " | 18.8 | .4 " | 25.4 | .8 " |
| 200 | 28.0 | 1.0 " | 16.5 | .3 " | 22.0 | .6 " |
| 250 | 22.6 | .6 " | 11.6 | .2 " | 18.3 | .4 " |

volume from 10-100 ml. Mice or small rats up to about 75 g and tissue samples can be counted in the plastic well without correction for geometry. The efficiency of 250 ml in the plastic scintillation counter is 22.6, 11.6 and 18.3% for $Co^{56}$, $Co^{58}$, and $Co^{60}$ respectively and 7.8, 3.5, and 3.8% respectively for 1000 ml using the beaker on the NaI well. The choice of instrument for counting large volumes is discussed.

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## Mechanism of Hypoglycorrhachia in Experimental Pneumococcal Meningitis.* (25356)

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It is well known that glucose in cerebrospinal fluid decreases in patients with bacterial meningitis, while non-bacterial meningitides are usually not associated with hypoglycorrhachia(1). The reason for this phenomenon is poorly understood particularly since it has been demonstrated that both leukocytes and bacteria are capable of accelerating consumption of cerebrospinal fluid in glucose *in vitro* (2,3). In our experiments pneumococcal meningitis was produced experimentally in dogs made leukopenic by total body irradiation. In these animals the leukocytic response in the CSF was effectively suppressed, and decrease in CSF glucose was also prevented despite heavy bacterial growth.

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TABLE I. Mean Values for CSF Leukocyte and Bacterial Counts and Glucose in 12 Irradiated and 13 Normal Dogs with Pneumococcal Meningitis.

| Hr after infection | WBC/mm$^3$ CSF | | Bacteria/mm$^3$ CSF | | Glucose/mm$^3$ CSF, mg % | |
|---|---|---|---|---|---|---|
| | Irrad. | Control | Irrad. | Control | Irrad. | Control |
| 24 | 490 (12)* | 4900 (13) | $2 \times 10^3$ (12) | $9 \times 10^2$ (13) | 90 (10) | 72 (12) |
| 48 | 530 ( 6) | 4000 ( 5) | $4 \times 10^4$ ( 6) | $6 \times 10^4$ ( 5) | 82 ( 6) | 50 ( 5) |
| 72 | 570 ( 4) | 7300 ( 4) | $8 \times 10^5$ ( 4) | $10^6$ ( 4) | 82 ( 4) | 52 ( 4) |

* No. of observations in parentheses.

*Methods.* Mongrel dogs weighing 8 to 12 kg were used. Bacterial meningitis was produced by inoculating a saline suspension of 1 ml of type III pneumococcus (containing $10^3$ to $10^5$ organisms) into the subarachnoid space through a small midline burr-hole placed equidistant between coronal and sagittal sutures. Cerebrospinal fluid was sampled by cisternal puncture 24, 48, 72 and 96 hours after induction of infection. Number of bacteria was determined by colony counts of serial 10-fold dilutions, a single colony being counted as one viable unit. Leukocytes were counted in standard manner and glucose was determined by glucose-oxidase method(4). Blood cultures were also made daily in all infected animals. Dogs were rendered leukopenic by administration of 450 r (total body irradiation) and used in experiments when their blood leukocyte counts had fallen to less than 1000/mm$^3$. In a group of control animals aseptic meningitis was produced by intrathecal administration of 1 ml of sterile normal dog serum. Seed cultures of type III pneumococci were stored at –70°C. To prepare a culture for infection of animals, the content of one tube was thawed and added to 10 ml of tryptose phosphate broth. After incubation at 37°C for 8 hours, the broth culture was diluted with 0.85% NaCl solution. Inocula consisted of 1 ml suspension containing $10^3$ to $10^5$ viable units. In order to minimize differences in virulence of individual cultures, each experiment was performed in leukopenic and normal (control) animals simultaneously.

*Results.* Pneumococcal meningitis was produced in 12 irradiated dogs; 13 animals served as controls. All irradiated animals had white blood cell counts of less than 1000/mm$^3$. In 21 animals the inoculum consisted of $10^5$ pneumococci; in 4, $10^3$ organisms were placed into the subarachnoid space. The course of the infection was the same regardless of size of the inoculum. The infection terminated fatally in 24 of 25 dogs; one animal in the control group recovered. Mean survival time of the leukopenic dogs was 60 hours following induction of the infection; control animals lived 55 hours. Bacteremia was present in 10 of 12 leukopenic animals and in 11 of 13 controls.

Average values of CSF leukocytes, bacteria and glucose of all animals are shown in Table I. The decrease in number of observations 48 and 72 hours after onset of infection is due to death in most animals and insufficient quantity of CSF in a few. Irradiated animals were capable of mobilizing only a small number of leukocytes in response to the meningeal infection, while the controls averaged close to 5000/mm$^3$ CSF. Despite this striking difference in CSF pleocytosis, the number of bacteria present in the CSF was approximately equal in both groups. As might be expected, the number of organisms increased on each day of the infection. There was considerable variation in individual dogs, some showing sudden sharp increments in number of bacteria as the infection progressed while in others the number of organisms remained relatively constant.

The normal mean for CSF glucose in this laboratory is 90 mg%, and the lower limit of normal 62 mg%. These results closely approximate those of Baltch and Osborne obtained by another method(3). The amount of glucose in the serum of dogs is usually slightly lower than that in CSF. Serum sugars were determined along with CSF sugars in all experiments, but since they showed no appreciable variation from normal are not detailed. The CSF of normal dogs with pneumococcal meningitis contained considerably less glucose

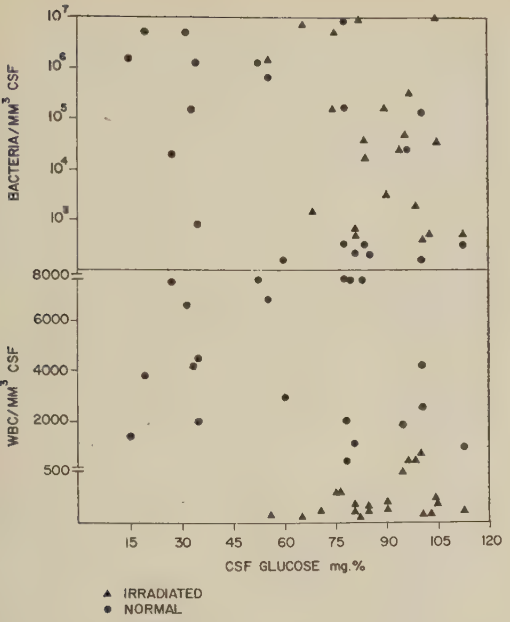

FIG. 1. Relationship between glucose, bacteria and leukocytes in spinal fluid. Glucose values in animals with low leukocyte counts were normal while 50% of animals with significant pleocytosis demonstrated hypoglycorrachia.

than that of irradiated animals (Table I). This difference became more apparent as infection progressed. On the other hand, no hypoglycorrhachia occurred in leukopenic dogs despite the presence of a large number of bacteria in the CSF. The relationship between number of leukocytes and bacteria and glucose in the CSF is illustrated in Fig. 1. Ten of 20 spinal fluids from normal animals with meningitis contained less than the normal amount of glucose; between 1600 and 9000 WBC/mm³ were found in each of these specimens. In contrast, only 1 of 21 samples of CSF obtained from leukopenic animals, the majority containing less than 500 WBC/mm³ of CSF, showed a lower than normal glucose level. Failure of glycolysis to occur in half of the infected controls remains unexplained; the infection in these animals did not differ from those in whom hypoglycorrhachia occurred. The inverse relationship between cells and sugar in the CSF was not apparent when the number of bacteria/mm³ CSF was compared with the glucose content, and the organisms appeared to grow equally well in the CSF of normal and leukopenic animals.

To ascertain whether CSF pleocytosis *per se* may result in a decrease in CSF glucose, nonbacterial meningitis was produced in 8 animals by intrathecal instillation of sterile, normal dog serum. These animals did not experience a fall in CSF glucose, but degree of pleocytosis was not as great as in the dogs with bacterial meningitis, averaging only 1000 WBC/mm³.

*Discussion.* These data indicate that suppression of pleocytosis in the CSF which occurs in response to a bacterial infection of the meninges is accompanied by absence of hypoglycorrhachia. This suggests that the leukocytes rather than bacteria are responsible for the decrease in CSF glucose in bacterial meningitis. This is probably not the case since glycolysis has been invariably absent in experimental aseptic meningitis even with cell counts as high as 13000(3). On the other hand, Goldring and Harford demonstrated *in vitro* that sterile cerebrospinal fluid from dogs with aseptic meningitis incubated at 37°C undergoes glycolysis, the decrease in sugar paralleling the number of cells(2). This contrasts sharply with the glycolytic activity of pneumococci which, when incubated with normal CSF, utilize glucose at a very slow rate. Indeed, enormous numbers of bacteria are necessary before the amount of glucose consumed approximates that metabolized by relatively few leukocytes in fluids from dogs with aseptic meningitis. Furthermore, the number of pneumococci necessary to lower CSF glucose *in vitro* in the absence of cells far exceeds that seen in experimental or clinical meningitis. Our studies confirm these observations *in vivo* since they demonstrate that bacteria growing in spinal fluid relatively free of cells do not produce a decrease in the fluid's sugar content. It remains to be demonstrated unequivocally, however, that bacteria growing in the presence of leukocytes utilize glucose better than those incubated in a cell-free medium.

One of the explanations advanced for failure to observe hypoglycorrhachia in aseptic meningitis is that the host is able to replenish CSF glucose more rapidly than it is metabolized by the leukocytes. Perhaps the irradiated dogs maintained CSF glucose close to normal by a similar mechanism since the radiation injury may have disrupted the blood-CSF barrier in some unknown fashion. There are no data which bear out this point. It is conceivable that leukopenia influenced the growth characteristics of bacteria in some unknown fashion to prevent utilization of glucose. This idea gains support in the observation that, *in vitro,* rapidly-growing pneumococci consume more glucose than organisms growing at a slow rate(2). Since only the absolute numbers of bacteria in the CSF were measured in our studies no information is available concerning their rate of multiplication.

These experiments do not provide the answer to the problem of hypoglycorrhachia in tuberculous meningitis which is usually characterized by relatively few cells and bacteria or in the occasional patient with pneumococcal meningitis with a large number of bacteria in the absence of significant pleocytosis. Nor do they shed light on the mechanism of transfer of glucose from the blood to the CSF. They do emphasize, however, that the mechanism governing the decrease in CSF glucose in bacterial meningitis is a complex one, and provide support for the argument that both cells and bacteria must be present for hypoglycorrhachia to occur.

*Summary.* Pneumococcal meningitis was produced in normal and irradiated dogs. The latter became leukopenic and the pleocytosis occurring in the CSF in response to the infection was suppressed. The number of bacteria in the CSF was the same in both groups, but CSF sugars remained consistently normal in the irradiated animals while hypoglycorrhachia occurred in 50% of controls. These findings suggest that both cells and bacteria are necessary to lower the CSF sugar in bacterial meningitis.

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## Liver Regeneration in Experimental Carbon Tetrachloride Intoxication.* (25357)

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Hepatic regeneration which follows carbon tetrachloride induced centrilobular necrosis has been assessed previously by histological (1), cytological(2-3), and chemical(4) means. These methods often yielded conflicting results and provided only limited insight into the process of liver cell proliferation. Availability of tritiated thymidine for detection of cells that are actively synthesizing DNA (5-6) made it possible to investigate carbon tetrachloride induced liver regeneration in more detail. Our purpose was to determine the time at which maximum regeneration occurs following a single dose of carbon tetrachloride; to localize the anatomical site and time sequence of parenchymal cell proliferation; and to attempt demonstration of a humoral factor responsible for hepatic regeneration after toxic liver injury.

*Material and methods.* Forty-five male Sprague-Dawley rats weighing 190-340 g were housed in individual cages and fed a standard diet of Purina Lab. Chow and water *ad lib.* Thirty-six rats were given 0.2 ml of $CCl_4$/100 g weight intragastrically *via* a polyethylene catheter; saline was given in the same manner

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to 9 controls. At intervals of 6, 12, 24, 36, 40, 48, 72, 96, and 120 hours after administration of carbon tetrachloride, 4 rats were injected intraperitoneally with 1 microcurie/g weight of tritiated thymidine‡ (specific activity 0.360 curie/millimol). Control rats were given labeled thymidine 8 hours after administration of saline. Experiments were scheduled so that tritiated thymidine was always given between 7 and 8 a.m. Four hours after administration of tritiated thymidine the animals were killed by decapitation and the liver removed, sliced and fixed in cold 10% neutral buffered formalin. After fixation tissue slices were washed in running tap water for 24 hours and then dehydrated, cleared and imbedded in paraffin. Sections of 7 to 8 $\mu$ thickness were prepared and stained with hematoxylin and eosin. Fine grain stripping film was applied over similar sections and exposed in a light tight box at 10°C for 30 days. The autoradiographs were developed in Kodak D-19 Developer for 7 minutes, fixed in Kodak D-19 Developer for 10 minutes, washed in cool running tap water for 1 hour and mounted in polyvinyl pyrrolidone. Estimation of mitotic activity and number of hepatic cell nuclei bearing the label was carried out as follows: for each liver, total number of hepatic cells present in an average of 10 (400 X) fields was determined in sections stained with hematoxylin and eosin. Fifty consecutive (400 X) fields were then searched for mitotic figures and results expressed/100,000 hepatic cells. In autoradiographs the number of labeled liver cell nuclei present in 100 consecutive (400 X) fields was counted and results expressed/100,000 hepatic nuclei. In 16 rats given orally 0.2 ml/100 g of carbon tetrachloride 42 hours earlier, whole blood was obtained at laparotomy under light ether anesthesia by cannulating the renal vein. Blood was heparinized and pooled and the plasma separated by centrifugation in the cold. Five ml of this pooled plasma was injected into a saphenous vein of each of 5 normal male rats. Five ml of pooled plasma obtained from 16 untreated animals was injected into each of 7 control recipients. Sixteen hours later recipients of plasma were given 1 microcurie/g of tritiated thymidine by intraperitoneal route and 4 hours later the animals were sacrificed. Histological sections and autoradiographs were prepared as described above.

*Results.* Histological changes in liver, consisting of a slight increase in intracellular fat and infiltration with inflammatory cells of the centrilobular area was detected 6 hours after carbon tetrachloride administration. At 12 hours these changes were more pronounced and at 24 hours marked centrilobular necrosis and inflammation involving ¼ to ½ of each lobule was present. Pyknotic nuclei, karyorexis and polymorphonuclear and monocytic cellular infiltration was noted. Intact liver cells were slightly larger than normal. At 6, 12 and 24 hours the number of hepatic mitoses was comparable to controls. Increased mitotic activity was first noted 36 hours after carbon tetrachloride and the mitotic index reached a maximum at 40 hours. Between 48 and 96 hours the degree of both necrotic and regenerative changes decreased and at 120 hours there was complete restoration of the parenchymal pattern without evidence of active necrosis or increased mitosis, mononuclear inflammatory cells being the only indication of prior liver injury.

The number of labeled hepatic nuclei in autoradiographs was similar in saline treated controls (Fig. 1) and in animals given tritiated thymidine 6 and 12 hours after receiving carbon tetrachloride. An increase in number of labeled hepatic nuclei was first noted at 24 hours and a maximum in labeling was reached at 40 hours (Fig. 2). The labeling of parenchymal cell nuclei was not limited to the centrilobular or periportal area but was diffuse and also involved the acinar region(7) and intermediate areas. In necrotic areas labeling was chiefly confined to nuclei of inflammatory and supporting tissue cells. At 48 and 72 hours the number of labeled nuclei was still markedly increased but a significant reduction in number occurred at 96 hours. At 120 hours labeling returned to control values. Comparative counts of mitoses and labeled nuclei showed a good correlation (Fig. 3) at each time interval after administration of carbon tetrachloride except at 24 hours when the

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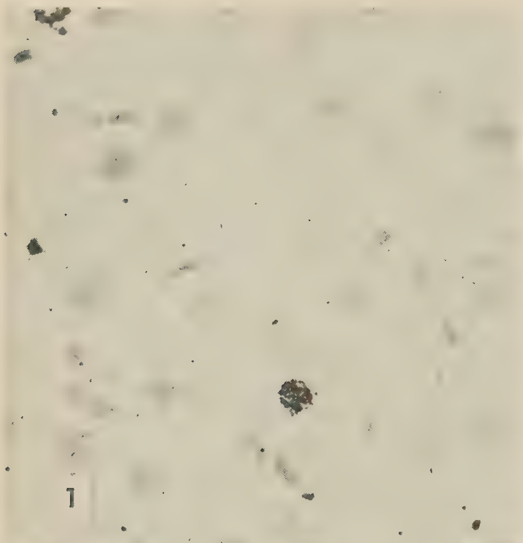
‡ Schwarz Labs., Mt. Vernon, N. Y.



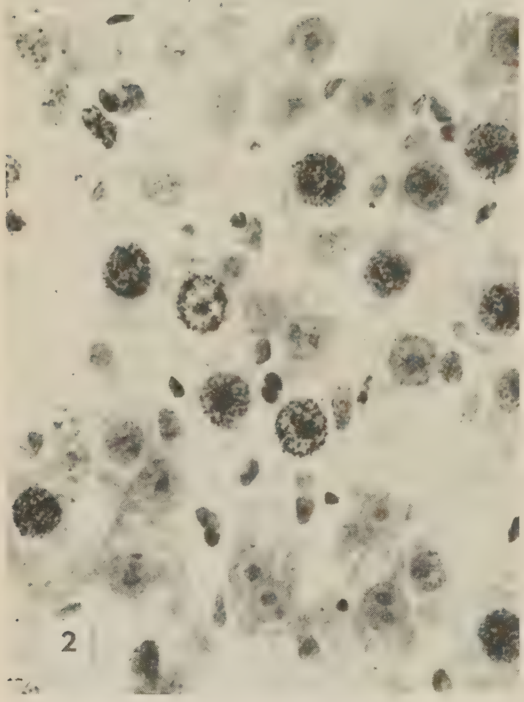


FIG. 1. Control rat liver, autoradiograph × 300. A single hepatic nucleus is labeled.

FIG. 2. Autoradiograph of liver 40 hr after a single oral dose of $CCl_4$ (400 ×).

average number of labeled nuclei increased to 282/100,000 cells while the mitotic index remained at control levels.

Pooled plasma from carbon tetrachloride treated rats had an albumin concentration of 2.65 g% and total protein 6.3 g% by chemical analyses(8,9); total serum bilirubin was 0.52 mg% by Malloy-Evelyn technic(10). Plasma from untreated rats had an albumin concentration of 2.15 g%, total protein 6.06 g%, and total serum bilirubin 0.12 mg%. There was no significant difference in number of mitoses and of tritium labeled hepatic nuclei between animals that had received plasma from rats treated with carbon tetrachloride and those given normal plasma. The carbon tetrachloride group had a mean average of 3.2 (S.D. ± 1.3) mitoses, and 44.6 (S.D. ± 21.8) labeled nuclei/100,000 liver cells; the controls had a mean average of 2.4 (S.D. ± 1.09) mitoses, and 28.4 (S.D. ± 12.2) labeled nuclei/100,000 liver cells. Neither group exhibited histological abnormalities in the liver.

*Discussion.* Carbon tetrachloride produces an increase in permeability of liver mitochondria(11), loss of diphosphopyridine nucleotides(12), and uncoupling of oxidative phosphorylation(13). This is associated with loss of hepatic glycogen(14) and co-enzyme A(15), and increase in neutral fat(14). A fall in level of nucleic acids during the period of necrosis(4) has not been confirmed by others(16). Our study shows that in early phases after carbon tetrachloride administration the number of labeled nuclei and mitoses is not less than that found in saline treated controls.

The importance of simultaneous histological, spectrophotometric, cytochemical, and chemical studies in evaluating the dynamics of

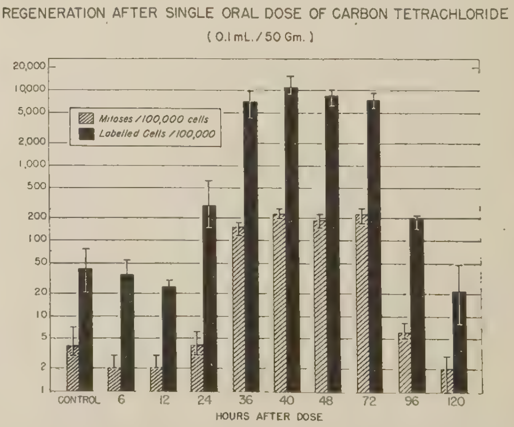


FIG. 3. Hepatic mitoses and labeled nuclei after single oral dose of 0.2 ml of $CCl_4$/100 g rat wt.

the regenerative process has been emphasized. This is illustrated by dissociation between mitotic counts and number of labeled nuclei at 24 hours after administration of carbon tetrachloride which probably reflects the increase in DNA synthesis occurring prior to actual cell division. Between 36 and 72 hours a concomitant increase in number of labeled hepatic nuclei and mitotic activity was noted (Fig. 3).

The magnitude and diffuse nature of the regenerative process in liver following carbon tetrachloride administration suggests that it may be due to other than local necrotic and reparative factors. Studies with partial hepatectomy in rats have demonstrated presence in plasma of a humoral principle for liver regeneration(17-21). In our study, plasma obtained from carbon tetrachloride poisoned rats at time of maximal liver regeneration failed to induce an increase in number of labeled cells or mitosis in livers of recipient animals. However, these preliminary results do not necessarily disprove the presence of a plasma factor for liver regeneration; changes in technic, use of plasma concentrates or variations in time between administration of $CCl_4$ and procurement of plasma or between injections of plasma and of tritiated thymidine might reveal the presence of a humoral principle which governs the repair phase following acute liver injury.

*Summary.* An autoradiographic technic employing tritiated thymidine was used to study liver regeneration in rats treated with a single oral dose of carbon tetrachloride. Results demonstrate that following toxic liver injury synthesis of desoxyribonucleic acid, indicating new cell formation, occurs chiefly in nonnecrotic areas of the liver; the regenerative process is diffuse and involves the acinar, intermediate, centrilobular and periportal regions. The highest rate of regeneration is reached 36 to 72 hours following administration of hepatotoxin, when the number of labeled liver cell nuclei is increased more than 250 fold over control values; after 120 hours, the regenerative process is virtually completed and the histological and autoradiographic pattern returns to normal. Under conditions of this experiment, the presence of a plasma factor for liver regeneration could not be demonstrated.

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# Effect of an Anion Exchange Resin on Serum Cholesterol in Man.[*] (25358)

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It is well known that bile acids are a major end product of cholesterol metabolism and that cholesterol is probably the only precursor of bile acids(1-3). Rate of oxidation of cholesterol to bile acids is believed to be regulated by need for bile acids for digestive purposes, cholesterol normally being oxidized at a rate sufficient to replace the bile acid that escapes reabsorption and is lost in the feces (4,5). If an agent could be found that would sequester bile acids and thereby promote their excretion in the feces, 2 consequences of clinical interest might be anticipated: (1) increase in rate of oxidative degradation of cholesterol, and (2) decrease in serum cholesterol level. Tennent(6) described lowering of serum cholesterol levels in animals fed the chloride salt of a basic anion exchange resin. This material (MK 135) has marked affinity for bile acids *in vitro* and is believed to exert its serum cholesterol-lowering effect by virtue of this property(6). The present report summarizes results obtained when MK 135 was administered to 26 patients, many with elevated serum cholesterol levels.

*Procedure.* Twenty-six patients were studied for 2 to 34 weeks. Fifteen patients had coronary artery disease; 3 essential hypercholesteremia; 4 normocholesteremia; and 4 moderately elevated serum cholesterol associated with mild, well-regulated diabetes mellitus. Four patients were studied while under treatment with acenocoumarin (Sintrom®). Patients ranged in age from 34 to 80 years. All except 3 were studied initially while in the hospital. Each patient continued with his previous diet and medications except that concurrent use of niacin (in pharmacologic amounts), sitosterols, estrogenic substances, and polyunsaturated fatty acid supplements was prohibited. Prior to administration of MK 135, at least 2 control measurements of concentration of serum total cholesterol and cholesterol esters were obtained. Subsequently, MK 135 was given to each patient in one of two forms: (1) as a liquid suspension containing 0.25 g of resin/ml, or (2) as a powder containing 0.83 g of resin/g of powder. Fifteen patients received 15 ml of the liquid 4 times a day (15 g of resin), and in 3 of this group, the dose was doubled during treatment. Eleven patients received 5 g of the powder 3 times a day (13 g of resin). The material was taken with or immediately after each meal and, when 4 doses were prescribed, at bed time. Serum total cholesterol and cholesterol ester levels were determined three times a week while patients were in the hospital and at weekly intervals as out-patients. Levels of total cholesterol and cholesterol esters were determined by the Schoenheimer-Sperry method(7). Patients were weighed frequently and a record was kept of dietary intake.

*Results.* In only 3 of the 26 patients were serum total cholesterol levels lowered by less than 10% (Table I). Average control levels for each individual ranged from 133 to 400 mg %, with average for group of 273 mg %. In terms of individual responses, average decrease in serum total cholesterol during treatment varied from 6.2 to 38.2%; in terms of the group, average decrease was 20.0 ± 8.0%[‡]. This was highly significant ($p < 0.001$). Multiple covariance analysis indicated that the patients with highest initial cholesterol levels tended to have greatest decreases ($p < 0.05$). In terms of clinical groups, average % decreases in level of serum cholesterol were as follows: familial hypercholes-

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[*] Supported in part by grant from Nutrition Fn., N. Y. City.

[†] Merck Inst. for Therapeutic Research.

[‡] $$\text{S.D.} = \sqrt{\frac{\Sigma\,(x - x)^2}{n-1}}.$$

TABLE I. Changes in Serum Total Cholesterol during Administration of MK 135.

| Diagnostic group | Avg initial serum total cholesterol (mg %) | Duration of treatment (days) | Avg % decrease in serum total cholesterol | Lowest level during treatment (mg %) |
|---|---|---|---|---|
| Coronary heart disease | 324 | 28 | 17.9 | 239 |
| | 270 | 21 | 19.1 | 207 |
| | 368 | 112 | 38.2 | 202 |
| | 320 | 22 | 28.3 | 198 |
| | 227 | 21 | 13.7 | 159 |
| | 254 | 49 | 9.5 | 207 |
| | 280 | 18 | 21.4 | 191 |
| | 259 | 16 | 26.6 | 185 |
| | 328 | 16 | 29.7 | 177 |
| | 243 | 28 | 14.4 | 160 |
| | 327 | 94 | 18.3 | 231 |
| | 209 | 14 | 7.7 | 172 |
| | 310 | 35 | 18.7 | 202 |
| | 272 | 14 | 22.1 | 170 |
| | 267 | 63 | 32.3 | 162 |
| Familial hypercholesteremia | 400 | 236 | 25.3 | 262 |
| | 352 | 32 | 25.0 | 264 |
| | 363 | 28 | 20.1 | 259 |
| Diabetes mellitus (mild) | 302 | 14 | 12.6 | 227 |
| | 232 | 18 | 10.3 | 191 |
| | 230 | 22 | 17.4 | 174 |
| | 307 | 21 | 20.8 | 200 |
| Normocholesteremic patients | 133 | 20 | 6.2 | 106 |
| | 208 | 16 | 11.3 | 174 |
| | 141 | 24 | 24.4 | 95 |
| | 168 | 20 | 29.3 | 91 |

terolemia 23.5%; coronary heart disease 21.2%; diabetics 15.3%; and "normocholesteremia" 18.1%. Serum cholesterol responses of representative members of each group to administration of MK 135 are shown in Fig. 1.

Six patients receiving MK 135 had only a slight decrease in serum total cholesterol while on the usual dose of 15 g of resin/day. When the dose was doubled 5 of these patients showed appreciable lowering in cholesterol level.

Three patients had complaints during MK 135 administration attributable to the medication. These included nausea and vomiting in one, nausea and constipation in one, and fecal impaction in one elderly, bed-ridden patient. Four additional patients volunteered the information that they had become moderately constipated while on the powder. No effect was noted upon appetite or weight nor was there impaired digestion as manifested by distention, diarrhea or bulky stools. Among the diabetic group and patients on acenocoumarin there were no changes in diabetic control or anticoagulant status.

Fifteen patients were followed after termination of MK 135 treatment. Eight showed a prompt rebound to levels ranging from 4.6

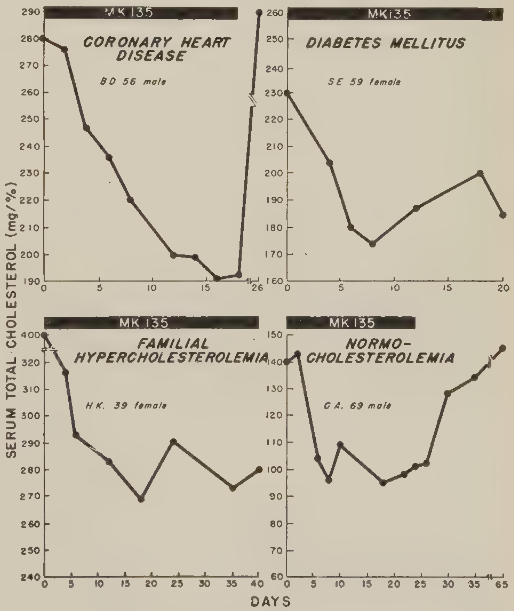

FIG. 1. Representative serum cholesterol responses to MK 135.

to 30.6% above control values. After 2 weeks, this "rebound" group returned to control levels.

Changes in esterified fraction of serum cholesterol always paralleled changes in serum total cholesterol.

*Discussion.* Although direct proof is lacking that the mechanism whereby MK 135 lowers cholesterol involves sequestration of bile acids, with increased cholesterol catabolism, animal experiments suggest that this is the most likely explanation(6). It was not possible in the present studies to assess rate of cholesterol catabolism or extent of fecal excretion of bile acids and other catabolic end products of cholesterol metabolism. Such studies are in progress.

The drop in serum total cholesterol levels after MK 135 treatment was begun usually was prompt, dramatic and sustained. Appreciable decreases in cholesterol level occurred within 4 days following MK 135 administration. Although the largest decrement occurred during first week of therapy, continued treatment was associated with further decreases during subsequent weeks. Six subjects were studied for 6 weeks or longer; all maintained lowered values of serum total cholesterol throughout the entire study period without evidence of "escape." Mean decrease of 20% was calculated by averaging cholesterol levels obtained in each subject at regular intervals following initiation of treatment and then using this value to determine mean decrease for the entire group. The lowest levels of serum cholesterol during treatment are shown for each subject in Table I.

Effectiveness of MK 135 is also indicated by its rate of success. Of 26 patients who were followed for a sufficient period, only 3 (or 11.6%) had a fall in serum total cholesterol of less than 10%. These 3 showed decreases of 6.2%, 7.7% and 9.5%. Hence, MK 135 was successful in reducing serum total cholesterol levels by more than 10% in approximately 88% of the series. This figure, as well as extent of decreases in cholesterol, compares favorably with results reported for other hypocholesteremic agents(8-10). It is also noteworthy that MK 135 was effective in lowering cholesterol in patients whose control levels of serum total cholsterol were already low by American standards.

Although analysis of dose-response curves was not done, there was an indication, within limits, of a direct relationship between amount of resin ingested and degree of depression of cholesterol levels.

In almost every case, MK 135 was well tolerated. Minor complaints such as constipation and nausea occurred in 6 patients (5 of whom were on the powder) and these disappeared promptly following discontinuance of treatment.

Following cessation of MK 135 administration cholesterol levels rose promptly. Of 15 subjects studied after termination of MK 135 treatment, 8 showed rises in cholesterol levels that transiently exceeded control values. Such an overshoot could have been anticipated on theoretical grounds since an enforced increase in the rate of cholesterol oxidation might be expected to result in some degree of increase in cholesterol synthesis.

*Summary.* Preliminary results of administering a resin with bile acid sequestering properties are reported. The preparation was administered to 26 patients for periods of 2 to 34 weeks. Serum total cholesterol levels were lowered by more than 10% in 23 of 26 patients. Average decrease in serum total cholesterol for all subjects was 20% ($p<0.001$). No systemic side-effects were observed. Six subjects treated for 6 weeks or longer maintained lowered values of serum total cholesterol without escape.

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# Absorption of Cholesteryl Methyl Ether and Its Effect on Cholesterol Absorption. (25359)

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Investigation of the role of esterification in absorption of cholesterol would indicate that during the absorptive process most of the cholesterol was esterified(1,2,3,4). However, Hernandez and associates(5) reported that epi-cholesterol, which is not esterified, was absorbed into the lymph of rats at about half the rate of cholesterol. This would suggest that esterification, although not obligatory, probably aids absorption. In addition, inhibition of cholesterol absorption by sitosterol is believed to be brought about by interference with its esterification(6). It was of interest to us therefore to study absorption of a sterol which could not be esterified. Accordingly, the methyl ether of cholesterol* was prepared and its absorption and effect on cholesterol absorption investigated in both rat and rabbit.

*Methods.* Cholesteryl-4-$C^{14}$ methyl ether was prepared by the method of Stoll(7) scaled down to mg quantities. In the final reaction, approximately 100 mg of p-toluene sulfonic acid ester of cholesterol-4-$C^{14}$ was refluxed in absolute methanol to yield 64 mg of the methyl ether of cholesterol-4-$C^{14}$ M.P. 81-82° Fisher M.P. block (reported 84°), specific activity (SA) 8300 cpm/mg. A paper strip chromatogram by the method of Hansen and Dam(8) indicated presence of a single spot (phosphomolybdic acid indicator) Rf 0.95 far removed from the cholesterol location, Rf 0.58, which coincided with the single peak of radio activity when analyzed on an Atomic Accessories chromatogram scanner. It is of interest that this compound was quantitatively precipitated by digitonin and gave essentially the same color value as cholesterol with $FeCl_3$-conc. $H_2SO_4$ color reagent(9). Unlabeled cholesteryl methyl ether to be used for diluting the labeled compound was prepared by the same procedure. This was analyzed for O-$CH_3$ and contained 7.51% O-$CH_3$; calculated 7.73%. The thoracic lymph duct of adult male rats, weighing 165-200 g, of Wistar strain, and which had been maintained on Purina Lab. Chow was cannulated by the Bollman technic(10). The animals were allowed only 0.9% saline *ad lib.* The following morning, those animals which exhibited good lymph flow (greater than 40 ml in 16 hours) were given, by intubation, 1 ml of an emulsion containing the labeled sterol. The emulsion, essentially that described by Swell and associates(4) with omission of glucose, was prepared by homogenizing 50 mg of labeled sterol, 50 mg egg albumin,† 150 mg of sodium taurocholate,† 0.16 ml oleic acid USP and 1.8 ml 0.9% saline. The final volume contained approximately 25 mg sterol and 90,000 cpm/ml. Lymph was collected with addition of heparin and stored at 5°C until analyzed. To determine overall recovery of $C^{14}$ activity, 0.1 ml samples of lymph were plated directly on aluminum planchets and counted in a Packard Flo-Window Counter. The remaining samples were lyophilized and pulverized for further work-up. A sample of lyophilized lymph was extracted with low boiling petroleum ether (30°-60°) by gentle refluxing for 1 hour. At least 80% of the radio activity was extractable by this method. An aliquot of the extract was applied to a silicic acid column for analysis of cholesterol esters, cholesteryl methyl ether and free cholesterol. This column was prepared as described(11), the extract applied and sterols eluted with chloroform-petroleum ether, 1:1. One ml fractions were collected, and analyzed for radio activity by direct plating and for sterol by the $FeCl_3$ colorimetric method(9). Male, Dutch Belted rabbits approximately 1.5 kg weight, were divided into groups of 6 and fed sterol supplemented diets. These diets were prepared by spraying Wayne rabbit pellets with an ether or chloroform solution of sterol and then air drying. Composition of diets is indicated in Table II. At weekly intervals blood samples from 3 rabbits/group were obtained for cholesterol analysis using the Trinder(12) saponification and extraction procedure, followed by $FeCl_3$ colorimetric method(9). At termination of experiment, the aorta including the arch and heart were examined grossly for evidence of plaque formation. These were scored from 0 to 3 depending upon involvement of the arch and thoracic aorta. The average of these scores gave the composite atheroma value.

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* $3\beta$ methoxy, 5 cholestene as designated in *Chem. Abst.*

† Obtained from Fisher Scientific Co.

*Results.* These studies may be divided into 2 aspects: absorption in the rat *via* lymph and absorption in the rabbit as a dietary supplement.

*Absorption of methyl ether of cholesterol-4-$C^{14}$ in the rat.* Table I shows recoveries obtained on oral administration of labeled sterol. The data indicate that the methyl ether of cholesterol was absorbed, although to a lesser extent than cholesterol.

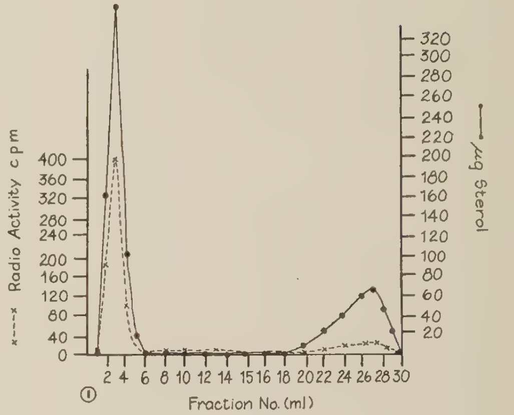
*Identity of sterol-$C^{14}$ in lymph.* Petroleum ether extracts of lyophilized lymph were analyzed on silicic acid columns as described above. Preliminary studies with authentic samples of cholesterol esters, cholesteryl methyl ether and free cholesterol located these compounds in the folowing 1 ml fractions: 2-5, 8-13, and 22-28 respectively. Typical chromatograms of lymph extracts from cholesterol-4-$C^{14}$ or cholesteryl-4-$C^{14}$ methyl ether fed animals are shown in Figs. 1 and 2 respectively. Cholesterol-4-$C^{14}$ appeared in lymph primarily in the esterified form. Appreciable dilution with endogenous cholesterol esters was observed since cholesterol administered possessed a SA of 3900 cpm/mg, while that recovered from lymph had a SA of 1300 cpm/mg. However, in the case of the methyl ether of cholesterol-4-$C^{14}$ there was no significant change in specific activity between administered and recovered compound.

TABLE I. Absorption of Sterols in the Rat as Measured by Recovery of $C^{14}$ Activity in Lymph.

| Sterol administered* | Lymph vol, ml† | % $C^{14}$ recovered |
|---|---|---|
| Methyl ether of cholesterol-4-$C^{14}$ | 106 (56-152) | 19.4 (16.9-24.6) |
| Cholesterol-4-$C^{14}$ | 107 (51-158) | 35.5 (31.6-37.9) |

* Administered orally, as an emulsion containing approximately 25 mg sterol and having 90,000 cpm. 4 rats/group.

† Twenty-four hr lymph collection. Values in parentheses are ranges.



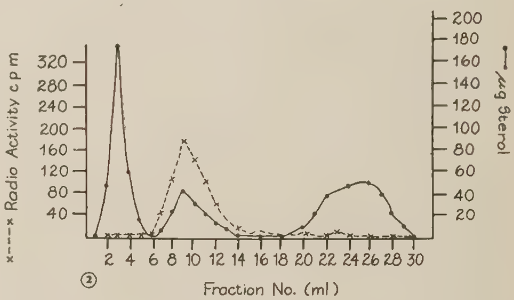


FIG. 1. Silicic acid column analysis of petroleum ether extract of lyophilized lymph from rat fed cholesterol-4-$C^{14}$ (SA 3900 cpm/mg). Fractions 2-5, cholesterol ester (SA 1300 cpm/mg). Fractions 20-30, free cholesterol (SA estimated at less than 300 cpm/mg).

FIG. 2. Silicic acid column analysis of petroleum ether extract of lyophilized lymph from rat fed the methyl ether of cholesterol-4-$C^{14}$ (SA 3700 cpm/mg). Fractions 7-12, cholesteryl methyl ether (SA 4000 cpm/mg).

*Effect of cholesteryl methyl ether on cholesterol-4-$C^{14}$ absorption in the rat.* Lymph duct cannulated rats were prepared as described and fed 1 ml of test emulsion by intubation. The emulsion was prepared as de-

TABLE II. Effect of Cholesteryl Methyl Ether on Cholesterol Absorption in the Rabbit.

| Group* | Diet | Avg serum cholesterol, mg % | | | | |
|---|---|---|---|---|---|---|
| | | 2nd wk | 4th wk | 6th wk | 8th wk | Atheroma‡ |
| A | 1% cholesteryl methyl ether | 46.0 ± 71† | 67.7 ± 15 | 62.3 ± 39 | 80 ± 64 | .1 |
| B | 3% cholesteryl methyl ether + 1% cholesterol | 542 ± 335 | 727 ± 329 | 685 ± 214 | 890 ± 245 | 1.75 |
| C | 1% cholesterol | 916 ± 763 | 1192 ± 940 | 1188 ± 885 | 1070 ± 406 | 2.4 |
| D | Pellets without supplement | 35.0 ± 10.3 | 29.6 ± 9.5 | 25.3 ± 15 | 30 ± 15.8 | .2 |

* 6 rabbits/group. † Stand. dev. ‡ Avg of both scores for the arch and thoracic aorta.

scribed above but modified so that each ml contained 10 mg cholesterol-4-$C^{14}$ (100,000 cpm) and 40 mg of cholesteryl methyl ether. Lymph was collected for 24 hours and recovery of radioactivity determined as described. In a group of 4 rats the average recovery of administered $C^{14}$ activity was 33.7% with a range of 27.5 to 38.8%. This data would indicate that as much as 4-fold quantities of cholesteryl methyl ether did not appreciably inhibit absorption of cholesterol.

*Effect of dietary cholesteryl methyl ether on cholesterol absorption in the rabbit.* This was studied by feeding rabbits a diet in which cholesterol and/or cholesteryl methyl ether was incorporated in pellets as described. Table II shows serum cholesterol values obtained during experiment and the final group atheroma index on termination. There is no significant difference between groups B and C in both serum cholesterol level and atheroma when evaluated by the Rank method of Wilcoxon(13). It is of interest to note that cholesteryl methyl ether alone had relatively slight effect on serum cholesterol.

*Discussion.* Absorption of cholesteryl methyl ether is an example of sterol absorption without esterification. Cholestenone(14) and epicholesterol(5) cited above, are other instances of this phenomenon which would indicate that esterification is not indispensable although it probably speeds absorption.

Studies in lymph duct cannulated rats indicated appreciable absorption of cholesteryl methyl ether and one would therefore expect significant elevation of serum sterol levels in the rabbit fed 1% cholesteryl methyl ether. That this did not occur may be indicative of a species difference between rabbit and rat with respect to absorption of cholesteryl methyl ether or that it may be rapidly metabolized or removed from the circulation soon after absorption. The answer to this question awaits further investigation.

Recently Blomstrand and Ahrens(15) reported cleavage of ether linkage of chimyl alcohol during absorption in man which confirmed earlier report in rats(16). This is of interest since no evidence of ether cleavage was noted in absorption of cholesteryl methyl ether reported here.

*Summary.* Oral administration of the methyl ether of cholesterol-4-$C^{14}$ to lymph duct cannulated rats resulted in approximately 20% recovery of $C^{14}$ activity in a 24 hour lymph collection as compared to 35% recovery in the case of cholesterol-4-$C^{14}$. Simultaneous administration of cholesteryl methyl ether with cholesterol-4-$C^{14}$ had no effect on cholesterol-4-$C^{14}$ absorption in the cannulated rat. In the rabbit, dietary cholesteryl methyl ether had negligible effect on sterol levels when given alone or in conjunction with cholesterol.

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## Pressures in the Common Hepatic Duct of the Rat. (25360)

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The first measurements of pressure in the common hepatic duct in the rat were reported by Mann and Foster(1), and the sphincteric action of the intraduodenal portion of the duct in animals including the rat was later investigated by Mann(2). Reports on pressure in the extrahepatic bile ducts of other animals followed(3-6), but measurements of the secretory pressure of bile in albino rats were not reported until Friedman, Byers and Michaelis (7) included observations on a small series as part of a study on production and secretion of cholesterol in mammals. In these studies pressures were measured with the water manometer. Kelsey and Beard(8) measured pressures in the common bile duct of human beings with a strain-gauge manometer. Since this manometer is more convenient for continuous recording than the water manometer, we repeated some of previous studies of hepatic duct pressures in the normal rat and studied also the pressure after certain induced hepatic injuries.

*Methods.* Adult albino rats of Sprague-Dawley strain were used. Rats weighed between 200 and 400 g. The rats selected for study were of 3 types: (a) rats with normal livers, (b) rats with hepatic cirrhosis induced by inhalation, in a closed chamber, of 25 mg of carbon tetrachloride/minute in 50 liters of air, for 6 hours, 3 times a week for 6 weeks and once a week for additional 4 weeks and (c) rats with passive venous congestion of the liver induced by application of a cellophane band around the inferior vena cava above the liver 4 weeks previously. The rats were fasted routinely for 16 hours before any observation of pressure was made. Under ether anesthesia the common hepatic duct of each rat in the study was isolated and ligated at its midpoint. Two lengths of polyethylene tubing (P.E. 50) with internal diameter of 0.58 mm were introduced into the duct; the first was directed toward the liver and the second toward, but not entering, the opening of duct into the duodenum. The 2 tubes were brought out through the skin. Free flow of bile into the duodenum could be restored by joining the ends of the tubes with a cuff of polyethylene tubing. After operation the rats were immobilized in a Bollman cage(9) and initial recordings of pressure were made at time intervals varying from a half hour to 16 hours after operation. During the intervening period the animals were allowed free access to drinking water. The pressure in the common hepatic duct was recorded by means of a strain-gauge manometer (Statham P6-4G-250, 0-4 P.S.I.) equipped with a 3-way stopcock. This system was filled with water, and all air excluded from the system before the test. The ends of the polyethylene tubes leading from hepatic duct, were connected to the stopcock, and the strain-gauge output was recorded on a photokymograph. The pressure transducer was referred to the level of the exit of the tubes through the skin of the animal, which closely approximated the openings

TABLE I. Mean Pressures in Common Hepatic Ducts of Albino Rats.

| | Flow pressure | | Duodenal-end pressure | | Secretory pressure | |
|---|---|---|---|---|---|---|
| Liver | No. observed | Mean pressures, cm $H_2O$ | No. observed | Mean pressures, cm $H_2O$ | No. observed | Mean pressures, cm $H_2O$ |
| Normal | 21 | 11.7 ± .8* | 14 | 6.4 ± .5 | 27 | 24.0 ± .7 |
| Cirrhotic | 9 | 14.3 ± 1.6 | 8 | 8.2 ± 1.5 | 9 | 29.2 ± 2.3 |
| Congested | 3 | 12.4 ± 1.6 | 3 | 9.1 ± .8 | 4 | 23.7 ± 2.5 |

* ± stand. error of mean.

of the tubes in the bile duct. The pressure recording system was calibrated at intervals by introduction of known levels of static pressure from a water manometer. By use of the 3-way stopcock the pressure in the tube directed toward the liver (secretory pressure), the pressure in the whole system (flow pressure) and the pressure in the tube directed toward the duodenum in the lower portion of the hepatic duct (duodenal-end pressure) could be measured. Recordings of each pressure were made for at least 15 minutes at each observation. As indicated by Kelsey and Beard(8), because of the small volume needed to register pressure changes in the strain-gauge manometer, the need for perfusion of the hepatic duct with saline solution is obviated and the pressures recorded represent, as far as possible, the pressures determined by secretory pressure of the liver and resistance of the bile duct. The liver and duodenal wall in the region of ductal orifice were examined histologically at conclusion of most studies. Five aspects of the problem were studied. Additional preparation of animals needed for some studies will be mentioned with results.

*Results. Measurements of flow, duodenal-end and secretory pressures.* Measurements of these pressures were made on rats with normal livers, with cirrhosis and with chronic passive venous congestion. Number of observations and mean pressures obtained are given in Table I. The secretory pressure was higher in cirrhotic rats than in rats with normal livers or with chronic passive congestion of the liver, and duodenal-end pressure was higher in rats with passive congestion of the liver.

Respiratory movements and duodenal muscular activity were transmitted by tubes in the hepatic duct and marked pressure changes were induced by movement or exaggerated respiratory excursions in the rat.

On histologic examination at conclusion of these studies, the normal group showed no abnormalities; cirrhosis and chronic passive venous congestion were the respective abnormal findings in livers of the other 2 groups of animals. Gross congestion of duodenal mucosa was found in animals with chronic passive venous congestion of the liver, but not in cirrhotic and normal rats.

*Simultaneous recording of biliary pressure and gastric and duodenal motility.* For this aspect of the study we used 3 rats with normal livers and 3 with cirrhosis. To record gastric and duodenal motility we introduced, at time of operation, polyethylene tubes (P.E. 160), of internal diameter 1.14 mm, into the gastric antrum and second part of the duodenum. The tips of tubes were covered by small latex balloons, and these and the tubes themselves were filled with water. The tubes were introduced into the stomach and duodenum through a small incision in the greater curvature of the stomach which was subsequently closed with a purse-string suture. The gastric and duodenal tubes were connected to strain gauges similar to those used for recording biliary pressure. The gastric and duodenal motility was recorded simultaneously with the biliary pressure by means of the photokymograph.

Although some fluctuations in recordings of biliary pressure could be correlated directly with bodily movement, respiration or gastric or duodenal activity, it was impossible to conclude that all variations were related to these factors (Fig.). No differences were noted in motility recordings between normal and cirrhotic rats.

*Biliary pressure after obstruction to the common hepatic duct.* All 3 types of rats were used for this aspect of the study. From each rat a base-line recording of secretory pressure was made for 2 hours. Subsequent to this the polyethylene tubing leading from upper por-

TABLE II. Mean Secretory Pressures in Common Hepatic Ducts of Rats after Complete Obstruction to Bile Flow.

| | Before obstruction | | Obstruction for: | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | 15-24 hr | | 40-48 hr | | 65-72 hr | | 92-137 hr | |
| Liver | No. observed | Mean pressures, cm $H_2O$ | No. observed | Mean pressures, cm $H_2O$ | No. observed | Mean pressures, cm $H_2O$ | No. observed | Mean pressures, cm $H_2O$ | No. observed | Mean pressures, cm $H_2O$ |
| Normal | 10 | 25.8 ± 1.3* | 11 | 23.9 ± 1.1 | 8 | 25.8 ± 1.6 | 3 | 24.7 ± 5.1 | 3 | 16.0 ± 6.0 |
| Cirrhotic | 4 | 27.8 ± .9 | 3 | 27.3 ± 2.6 | 2 | 24.4 ± 1.6 | 1 | 19.8 | 2 | 19.1 ± 7.2 |
| Congested | 4 | 24.5 ± 2.7 | 4 | 19.2 ± 3.3 | 3 | 19.9 ± 4.8 | 1 | 22.5 | 3 | 8.3 ± 1.8 |

* ± stand. error of mean.

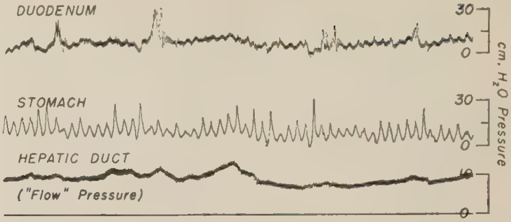

FIG. 1. Simultaneous recording of flow pressure in common hepatic duct and of movements of stomach and duodenum.

tion of common hepatic duct, was occluded by a clip to produce complete obstruction of outflow of bile, since in the preparation of the animal a ligature had already been applied to the midpart of the duct. Further recordings of secretory pressure were made at intervals after the occlusion. The 3-way stopcock obviated release of obstruction at any time. Each recording was of 15 to 20 minutes' duration. Sixty-two observations of secretory pressure were made on this group, 18 before obstruction and remainder at intervals from 15 to 137 hours after obstruction was begun. Eight were made during obstruction of 92 to 137 hours. These secretory pressures and the number of observations are summarized in Table II. Secretory pressures of animals tested showed no statistically significant alterations during obstruction to the common hepatic duct.

All rats became deeply jaundiced, however, and at necropsy gross dilatation of the extrahepatic bile ducts above the ligature was found. In no instance could leakage of bile into the abdominal cavity be demonstrated. On histologic examination extensive focal cellular necrosis of the liver was seen in all animals, but gross evidence of cholestasis was found only in cirrhotic livers.

*Biliary pressure after inhalation of carbon tetrachloride.* After control pressure readings were obtained, 6 rats with normal livers were placed in a closed chamber for 6 hours where they would inhale 25 mg of carbon tetrachloride/minute in 50 liters of air. Secretory pressure was recorded immediately and 24 hours after removal from the chamber. Two rats survived so that recordings could be made immediately after a second exposure for 6 hours to carbon tetrachloride. Each recording lasted half an hour. Free flow of bile into the duo-

TABLE III. Mean Secretory Pressures in Common Hepatic Ducts of Rats after Inhalation of Carbon Tetrachloride.

| | No. observed | Mean pressure, cm $H_2O$ |
|---|---|---|
| Control | 6 | 23.8 ± 1.1* |
| After inhalation of carbon tetrachloride | | |
| Immediately | 6 | 21.7 ± 1.1 |
| 24 hr later | 5 | 20.4 ± .7 |
| Immediately after 2nd exposure | 2 | 21.5 ± 1.4 |

* ± stand. error of mean.

denum was permitted during periods of inhalation and between recordings. Secretory pressure was slightly lower after inhalation of carbon tetrachloride. The pressure findings are summarized in Table III.

*Biliary pressure after intravenous administration of chlorpromazine.* Five rats with normal livers were used. Initial pressure readings were made and then 10 mg of chlorpromazine hydrochloride (thorazine)/kg of body weight was injected *via* tail vein. Continuous recordings were made for 3 hours after injection. For 2 rats, injection of thorazine was repeated at the end of 3 hours, and further pressure recordings were made for a similar period. Intravenous injection of thorazine was followed in all 5 rats by diminution in duodenal activity as shown by transmitted movement recorded from the tubes in the hepatic duct. This commenced within 5 minutes of injection and its duration was approximately 1 hour. All rats became drowsy within 5 minutes, and one animal had a generalized epileptiform convulsion lasting 2 to 3 seconds immediately following injection.

No effects on secretory, flow, or duodenal-end pressures were found in any animal in the 3-hour period following first injection or in the 2 rats given second doses. All livers were examined histologically at conclusion of experiments, and no changes could be found.

*Comment.* The mean level of secretory pressure of bile in the fasting rat with normal liver in this series, is broadly in agreement with that recorded by Mann and Foster(1) and by other investigators(7,10,11). Increase in secretory pressure in the cirrhotic rats over that in animals with normal livers and in those with chronic passive congestion of the liver is statistically significant (Table I). The mean flow pressure of 11.7 ± 0.8 cm of water in rats with normal livers lies within the range given by Kelsey and Beard(8) who, in the human subject, noted that this pressure varied from 7 to 22 cm of water. No previous recordings of flow pressure of bile in the rat are available for comparison.

From simultaneous recordings of biliary ductal pressure and gastric and duodenal motility, it was concluded that, in the albino rat, some sphincteric action is exerted by the lower end of the common hepatic duct and that, in normal and cirrhotic rats, the duodenal-end pressure can be interpreted as that sustained by the resistance of bile duct and the sphincteric action of its intraduodenal portion. This concept was suggested by lack of direct correlation between all fluctuations in recordings of ductal pressure and recordings of duodenal activity. If the duodenal-end pressure simply reflected intraduodenal pressure such a correlation would be expected. Existence of an anatomic sphincter has been observed in the rat(2). The higher duodenal-end pressure found in animals with chronic passive venous congestion of the liver, probably is related to congestion of the duodenal tissues near the intraduodenal portion of the common hepatic duct in these rats.

The apparent fall in mean secretory pressure in the 8 rats in which obstruction to the common hepatic duct had been maintained for 92 to 137 hours is considered to reflect inanition of these nearly moribund animals.

The fall in secretory pressure in rats on inhalation of carbon tetrachloride vapor was significant on statistical analysis. No previous findings in the rat are available for comparison.

Absence of any change in flow or duodenal-end pressures in the group which received thorazine by intravenous injection is in contrast to those of Menguy, Grindlay, and Cain (12) who found a rise in ductal pressure in cholecystectomized dogs after intravenous injection of the drug. No change in ductal pressure, however, was noted by Kelsey and Beard(8) in human subjects who were given thorazine by mouth.

*Summary.* 1) A method for recording pressure within the common hepatic duct of the rat with a strain-gauge manometer is described. Measurements of pressure in the common hepatic duct were made on a series of fasting rats with normal livers, cirrhosis or passive venous congestion of the liver, and these were analyzed statistically. The higher mean secretory pressure in the rat with cirrhosis over that in the rat with normal liver and with passive venous congestion is statistically significant. 2) Secretory pressure in rats after inhalation of carbon tetrachloride vapor fell slightly but significantly. 3) The duodenal-end pressure in the common hepatic duct was related to findings on simultaneous recordings of biliary pressure and gastric and duodenal motility. This pressure was significantly higher in rats with chronic venous congestion of the liver than in normal or cirrhotic animals. The lower end of the common hepatic duct probably has some sphincteric action in the rat. 4) Intravenous injection of chlorpromazine hydrochloride (thorazine) produced no effects on pressure in the common hepatic duct in normal rats.

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## Studies on Erythropoietin. II. Production of High-Titer Plasma in Sheep.*

(25361)

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Most investigators used rabbits for production of erythropoietin-containing plasma, although the literature suggests that ability to respond to various erythropoietic stimuli is widespread among mammals. At start of our program aiming toward isolation and clinical testing of the pure factor, it became clear that an animal considerably larger than the rabbit would be desirable for economical production of amounts of material needed. After careful consideration of the price and availability of various domestic animals, we selected cull breeder sheep as most suitable for our needs.

*Methods and materials.* Cull breeder sheep, mostly ewes, in age range of 3 to 7 years, and weighing over 100 lb. (45.5 kg) were used. Hay constituted the bulk of diet during experiments, and water was supplied *ad lib.* Anemia was induced by subcutaneous injection of phenylhydrazine† above shoulder, alternating sides, on days 1, 3, and 5. Blood was harvested by exsanguination on day 7, using the following procedure: the animal was shackled by hind feet and lowered into a plastic bag containing an atmosphere of $CO_2$ supplied by dry ice. When struggling ceased (approximately 20 seconds), an incision through the skin was made with sharp knife starting from chin and extending to breast

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* This article is based on work done under contract with Atomic Energy Comm.

† For injection, neutralized phenylhydrazine hydrochloride (Fisher Certified Reagent) was used at a concentration of 24 mg/ml.

TABLE I. Blood Values and Potency of Plasma of 16 Cull Breeder Sheep Given 48 mg Phenylhydrazine/kg Body Weight.

| | Hematocrits | | | | Potency of plasma (u/ml) |
|---|---|---|---|---|---|
| Wt (kg) | Initial | Day 3 | On drawn blood | Blood vol (l) | |
| 67 | 35 | 33 | 25 | 2.0 | .34 |
| 71 | 33 | 14 | 7.0 | 1.9 | 1.6 |
| 79 | 39 | 31 | 13.5 | 1.7 | 1.8 |
| 41 | 40 | 35 | 33 | 1.7 | .10 |
| 74 | 36 | 26 | 15.5 | 1.3 | .86 |
| 52 | 42 | 30 | 21 | 1.7 | .32 |
| 47 | 35 | 31 | 22 | 1.6 | .61 |
| 72 | 34 | 26 | 12.5 | 1.8 | .88 |
| 61 | 53 | 37 | 23 | 1.1 | .44 |
| 72 | 41 | 30 | 8.0 | 1.5 | 7.4 |
| 38 | 48 | 32 | 17 | 1.3 | .52 |
| 77 | 37 | 13 | 9.0 | 1.6 | 3.5 |
| 88 | 35 | 18 | 10.5 | 2.2 | 3.6 |
| 92 | 42 | 31 | 23 | 3.1 | .41 |
| 59 | 32 | 21 | 6.5 | 1.9 | 5.9 |
| 97 | 37 | 22 | 13.5 | 2.1 | 1.2 |

bone. Retractors were inserted to spread the wound. By a stainless steel knife welded into a stainless steel funnel, both aorta and jugular were severed by a sharp upward thrust into thoracic area. The funnel directed the flow of blood through a plastic tube into a plastic vessel containing heparin. The bleeding process required one to two minutes. The cells were removed from blood by centrifugation in refrigerated Sharples laboratory super-centrifuge. During the process, temperature of plasma was reduced to 5-10°C. Plasma samples were stored frozen and assayed by procedure described previously(1).

*Results.* In preliminary experiments, the dose of phenylhydrazine was based solely on body weight of animals. In accordance with the work of Jacobsen, *et al.*(2), on rabbits, the sheep were given a total of 48 mg phenylhydrazine hydrochloride/kg body weight in 3 equal doses on days 1, 3, and 5. All animals were bled on day 7. Table I shows the results. Animals varied considerably in their initial hematocrit levels and in their reaction to standard dose of phenylhydrazine. More importantly, the potency of plasma varied greatly, ranging from 0.1 to 7.4 units/ml. The very high potencies obtained in plasma from animals with low hematocrit values suggested a logarithmic relationship and, indeed, the best straight-line fit was obtained by plotting on semi-log paper, as shown in Fig. 1. This relationship showed the need for obtaining low hematocrit values in the sheep before harvesting blood. Accordingly, all practical measures were taken to attain hematocrit levels in the range of 10-15% at time of blood harvest (7th day). This was done by the following procedure.

The first and second doses (on days 1 and 3) were equal and based on body weight, with a downward adjustment for subnormal initial hematocrit values. For animals with hematocrits of 40 or over, the first 2 injections were each 16 mg phenylhydrazine hydrochloride/kg body weight. Doses for animals of lower hematocrits were scaled downward proportionately until a level of 10 mg/kg was reached at hematocrit level of 30. On day 5, another hematocrit value was taken and size of third injection was then based on progress of animal's blood level toward the target value of 10-15% hematocrit on day 7. The status on day 5 could be judged quickly by making a rough plot of hematocrit readings against time and then comparing the slope of the straight line connecting initial and 5th day values with the slope obtained by drawing a

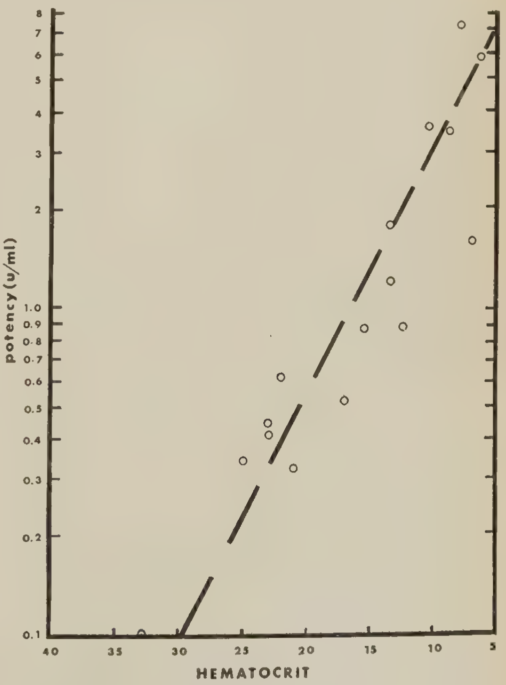

FIG. 1. Potencies of sheep plasmas plotted against final hematocrit values.

TABLE II. Blood Values of 24 Sheep Given Adjusted Phenylhydrazine Dose.

| Wt (kg) | Initial hct | Hct on day 5 | Factor for 3rd dose (day 5) | Final hct |
|---|---|---|---|---|
| 41 | 32 | 21 | 1.0 | 11 |
| 55 | 38 | 33 | 2.0 | 19 |
| 52 | 32 | 25 | 1.0 | 9 |
| 91 | 43 | 28 | 1.25 | 19 |
| 55 | 39 | died | | |
| 77 | 44 | 25 | 1.0 | 9 |
| 64 | 39 | 29 | 1.5 | 16 |
| 64 | 38 | died | | |
| 71 | 39 | 22 | 1.0 | 13 |
| 82 | 47 | 24 | .75 | 15 |
| 51 | 36 | 24 | 1.25 | 18 |
| 113 | 45 | 27 | .75 | 11 |
| 50 | 43 | 21 | .75 | 8 |
| 62 | 40 | 27 | 1.5 | 22 |
| 66 | 39 | 17 | .15 | 12 |
| 66 | 38 | 24 | 1.25 | 9 |
| 82 | 41 | 20 | .75 | died |
| 57 | 37 | 16 | .15 | 13 |
| 50 | 42 | 20 | .4 | 9 |
| 71 | 34 | 24 | 1.25 | 13 |
| 114 | 41 | 18 | .1 | 13 |
| 75 | 29 | 28 | 2.0 | 17 |
| 109 | 41 | 27 | 1.25 | died |
| 91 | 35 | 31 | 2.0 | 10 |

line from initial hematocrit value to 12.5 at day 7.

The results of a representative experiment are shown in Table II. A total of 31 liters of plasma was obtained from 20 animals which survived until blood harvest. The potency of the pool was 1.25 units/ml, giving a total of almost 40,000 units. Similar pools from 50 animals have varied in potency from 0.6 to 2.5 u/ml, with an average of 1.3.

*Discussion.* The logarithmic relationship suggested by Fig. 1 indicates that very effort should be made to induce low hematocrit levels. In a practical way, however, the benefits are balanced by losses incurred by deaths, as dose of phenylhydrazine is increased. We found that increasing the dose even 20% beyond that given above, results in a marked increase in mortality and the resulting decrease in plasma volume is not compensated by the increase in potency.

Another variable which we have considered is rate at which anemia is induced. Previous investigators, including Jacobsen, *et al.*(2), used approximately one week. From an economic standpoint, it would be desirable to decrease this period. However, it has been our experience that plasma from animals brought to low hematocrit levels in 4 days by injection of larger doses of phenylhydrazine has been much less potent than that from animals brought to comparable blood values in the usual 7 day period.

Goldwasser, *et al.*(3) have shown that injection of cobalt into rats markedly increases the erythropoietin titer of plasma. We obtained a similar effect in sheep. However, blood from cobalt-treated sheep contains such a large volume of cells that only about 40% of the volume is recoverable as plasma, while blood from phenylhydrazine-treated sheep yields 80% as plasma. Since cobalt plasma was not more potent than phenylhydrazine plasma, the use of cobalt was eliminated on an economic basis.

It has been estimated(1) that normal sheep plasma contains only about 0.01 u/ml of erythropoietic activity. Thus increases produced during the phenylhydrazine process are of the order of several hundred times. Since no storage organ for erythropoietin has as yet been discovered, it appears that it is all produced during the 7 day period with the greater part being made in the last 24-48 hours. At least one disease condition in humans gives rise to plasma concentrations comparable to the highest levels produced in our sheep experiments. We have recorded values as high as 8 u/ml in hypoplastic anemia patients. Others(4) have reported similar high levels.

*Summary.* Plasma of high erythropoietic titer has been produced in cull breeder sheep by the use of phenylhydrazine. The potency of plasma appears to have an inverse logarithmic relationship to the final hematocrit levels of the sheep blood. By making adjustments of the phenylhydrazine dose according to the condition of the individual sheep, the potency of the plasma pools from 25-50 animals has been raised to an average of 1.3 u/ml. This value is about 130 times the potency of normal sheep plasma.

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# Effects of Protein Depletion and Repletion on Serum Electrophoretic Patterns of Adult Rats.[*] (25362)

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A number of studies concerned with effects of nutritional states on serum proteins of several species have been reported(1,2). Serial determinations of serum proteins during protein depletion and repletion, however, have been limited to a great extent to man and the dog, and there is some evidence of species differences in their response(2). In the present study, partition of serum proteins has been determined by filter paper electrophoresis in groups of adult rats sacrificed at intervals during protein depletion and repletion.

*Methods.* Male, Sprague-Dawley rats were housed singly in suspended type wire cages with wire bottoms and maintained on a diet of Purina Lab. Chow and tap water, *ad lib.* until initiation of the study. Blood samples were obtained by exsanguination from the heart under ether anesthesia. *Normal.* Two normal groups were employed. Group A1 served as base line control and A2 as age and weight control. They were maintained under conditions described above. *Depletion.* The animals were fed protein-free diet[†](3) until the desired weight loss was achieved. Groups of rats were sacrificed after losing 10, 25, 33 and 40% of original weight. *Repletion.* Purina Lab. Chow with protein content of approximately 25% was fed *ad lib.* to animals with 25% weight loss. In an earlier study(4), the diet was adequate for weight and serum protein repletion. Groups of rats were sacrificed after regaining 35, 70 and 100% of their lost weight. One group, C4, was allowed to survive 4 weeks following weight repletion. *Chemical and hematologic analyses.* Total serum protein, hemoglobin and hematocrit values were determined by methods previously reported(3). Electrophoretic analyses were carried out by procedures previously reported (5) except that a current of 6 ma was employed. Use of a slightly higher amperage resulted in better resolution of the albumin and $\alpha_1$-globulin fractions. Analysis of the data was made by standard statistical procedures employing the $t$ test(6).

*Results.* General and hematologic data are summarized in Table I. Plasma volume as indicated by hematocrit values, fluctuated during protein depletion and was significantly increased in repletion. Relative concentration changes of serum fractions, however, were not significantly affected. Hemoglobin was depleted and restored at a slower rate than the serum proteins. Four weeks following repletion (C4), hemoglobin and hematocrit values were in the normal range. Body weight curves during protein depletion and repletion are presented in Fig. 1. The curves were characterized by marked inflections following dietary change.

Results of electrophoretic analyses are summarized in Figs. 2, 3, and 4. Feeding a protein-free diet for one week (B1) caused highly significant decreases in total protein, albumin and $\beta$-globulin concentrations. Moderate depletion (B2) elicited significant decreases in total protein, albumin and the $\alpha$-globulins

TABLE I. General and Hematologic Data.

| Group | No. rats | Hemoglobin (g %)* | Hematocrit (%)* |
|---|---|---|---|
| A1 Normal (400 g) | 14 | 14.1 ± .10 | 44 ± .4 |
| A2 " (460 g) | 14 | 13.8 ± .20 | 46 ± .5† |
| B1 10% depleted | 12 | 14.7 ± .15† | 49 ± .3† |
| B2 25% " | 12 | 14.1 ± .40 | 43 ± .4 |
| B3 33% " | 9 | 11.4 ± .36† | 36 ±1.4† |
| B4 40% " | 9 | 11.8 ± .33† | 42 ±1.7 |
| C1 35% repleted | 12 | 11.8 ± .24† | 38 ± .6† |
| C2 70% " | 12 | 11.8 ± .30† | 37 ± .5† |
| C3 100% " | 12 | 11.9 ± .24† | 38 ± .6† |
| C4 4 wk post repletion | 26 | 14.0 ± .20 | 45 ± .5 |

* Including stand. error of mean.

Statistically significant differences from baseline values (A1) are indicated:
† P = <.01.

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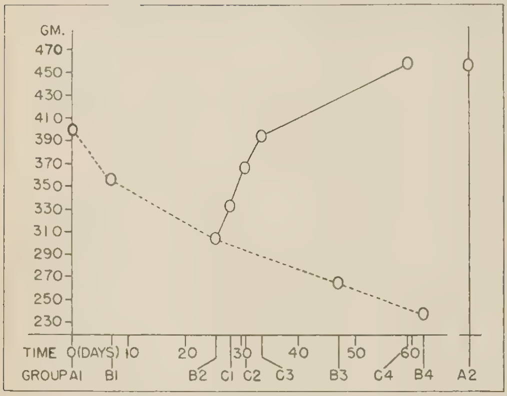
[*] Supported in part by research grant from Nat. Cancer Inst.

[†] Obtained from Nutritional Biochemicals Corp., Cleveland.



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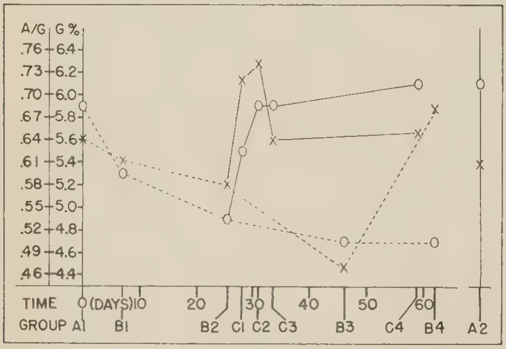

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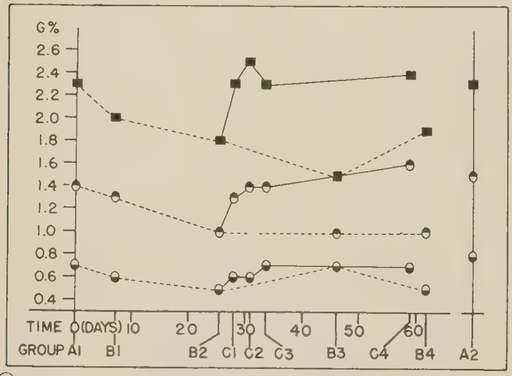

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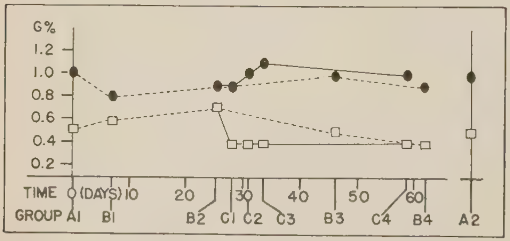

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FIG. 1. Effects of protein depletion and repletion on body wt. Dashed and solid lines indicate when protein-free and stock diets, respectively, were fed.

FIG. 2-4. Effects of protein depletion and repletion on serum protein concentrations. Dashed and solid lines indicate when protein-free and stock diets, respectively, were fed. Plotted values are not corrected for experimental hematocrit. 2. ○, total protein; ×, A/G ratio. 3. ■, albumin; circle (solid top), $\alpha_1$-globulin; circle (solid bottom), $\alpha_2$-globulin. 4. ●, $\beta$-globulin; □, $\gamma$-globulin.

concomitant with a significant increase in $\gamma$-globulin. Further depletion was characterized by divergent responses in subfractions of serum. The greatest changes were in the albumin fraction which resulted in marked alterations in A/G ratios (*cf.* B3, B4). At end of the depletion phase (B4), decreases in total protein, albumin, $\alpha_1$ and $\alpha_2$-globulin values were significant at the 1% level; those for $\beta$- and $\gamma$-globulins at the 5% level.

Rapid increases occurred in major serum components upon introduction of the stock diet (C1). Total protein, albumin, $\alpha_1$–, $\alpha_2$– and $\beta$-globulin concentrations were restored to their normal range before weight repletion (C2). At weight repletion (C3) and 4 weeks following repletion (C4), the differences between $\gamma$-globulin values for the experimental and control groups (A1, A2) were significant at the 5% level. Comparison of the 2 normal groups (A1, A2) showed no significant differences in distribution of serum proteins with increasing age and weight.

*Summary.* Distribution of serum proteins has been determined by filter paper electrophoresis in groups of adult rats sacrificed at intervals during protein depletion and repletion. In the early stages of protein depletion and repletion, body weight changes were paralleled to a considerable extent by similar alterations in serum protein levels. Such correlation was not apparent during later stages. The albumin and $\alpha_1$-globulin fractions were most sensitive to nutritional influences. The $\gamma$-globulin fraction deviated from the general pattern of response.

The technical assistance of Dorothy Roberts is gratefully acknowledged.

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# Tolerance of the Profoundly Hypothermic Dog to Complete Circulatory Standstill.* (25363)

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(Introduced by B. Woodhall)
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Introduction of a clinical method of inducing and reverting hypothermia(1) has made determination of safe limits of complete circulatory standstill at 10°C or lower of practical importance. Experiments outlined below represent our efforts to define these safe limits.

*Methods.* Healthy mongrel dogs were anesthetized with intravenous sodium pentobarbital. Extracorporeal circulation and hypothermia were carried out by a pump-oxygenator-heat-exchanger device already described (1). Just before perfusion, 28-30 mg/kg of quinidine hydrochloride was given intravenously. The mid-esophageal temperature, as measured by a thermistor, was rapidly reduced to between 5°C and 9°C. At this point circulatory standstill was permitted by stopping the pump. Temperature drifts during cessation of circulation were prevented by external application of ice. The chest was opened only when electrical defibrillation of the heart was needed.

*Experimental design.* The period of complete circulatory standstill and the number of dogs in each period were as follows: 30 min., 2 dogs; 60 min., 12 dogs; 90 min., 6 dogs; 120 min., 2 dogs; 180 min., 2 dogs. Surviving animals were followed for at least 7 days and observed closely for central nervous system changes.

*Results.* The 14 dogs in the 30-minute and 60-minute groups all survived 7 days or longer and were completely normal. In the 90-minute group, 4 survived and were normal; but 2 of the survivors developed ventricular fibrillation that had to be corrected by electrical defibrillation. The remaining 2 in this group, as well as the 4 dogs in the 120-minute and 180-minute groups, died from irreversible ventricular fibrillation that developed in the rewarming period.

*Discussion.* Temperatures below 10°C induced and reverted with an extracorporeal system, permit 60 minutes of safe complete circulatory standstill in dogs. Periods of 90 minutes are less well tolerated, as shown by occurrence of ventricular fibrillation. This arrhythmia was irreversibe in 4 dogs in circulatory standstill for more than 90 minutes, as well as the 2 in the 90-minute group. Development of ventricular fibrillation may indicate that rate of metaboism in the heart, even though greatly reduced by the cold and the asystole, is still sufficient to cause irreversible changes if oxygen deprivation is continued beyond 60 minutes.

*Summary.* Periods of 60 minutes of complete circulatory standstill were tolerated in 10 dogs at temperatures of 10°C or lower.

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# Catecholamine Production in Tissue Cultures of Human Adrenal Medullary Tumors and of Adrenal Medulla.* (25364)

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While metabolism of catecholamines of the adrenal medulla has been extensively investigated in various animal species *in vivo* or by utilizing tissue slices or cell fractions *in vitro* (1), no studies of hormone production in tissue cultures of adrenal medullary cells have previously been reported.

*Methods.* The material for tissue cultures was obtained from 2 cases of pheochromocytoma removed in University of Virginia Hospital last year. The first patient was a 24-year-old white female. Her urinary catecholamine excretion before operation was 651 μg noradrenaline (NA)/24 hr and 364 μg adrenaline (A)/24 hr. A pheochromocytoma of the right adrenal weighing 153 g and measuring 9 x 6 x 5 cm was removed on July 28, 1958. One week after the operation, the patient excreted 28.8 μg NA/24 hr and 20.7 μg A/24 hr. The second patient was a 29-year-old white female with preoperative urinary catecholamine excretion of 670 μg NA/24 hr and 451 μg A/24 hr. A pheochromocytoma of the left adrenal, removed May 14, 1959, weighed 63 g and measured 6 x 6 x 4 cm. The bloody fluid which exuded from the tumor and from the adrenal into the Petri dish in which the specimen was transported to the laboratory contained more than 1675 μg NA/ml and 1950 μg A/ml. One week after the operation, catecholamine excretion was 170 μg NA/24 hr and 28.4 μg A/24 hr. It returned to normal after 1 month (8.6 μg NA/24 hr and 3.6 μg A/24 hr). Explantations of tissues were started within 5 minutes after removal of tumor. Fragments of the tumor and of tumor-free parts of the adrenal medulla were grossly separated from the cortex and trypsinized for 10 min. before explantation into 40% human serum-Hank solution medium containing penicillin and streptomycin. During the first days the culture media became deeply colored by a brown-black pigment which may have been melanin. The explants both of tumors and of adrenals showed outgrowths of elongated large cells, 360-400 μ long, with large nuclei and nucleoli, 3 to 5 days after explantation. The cytoplasm contained a large number of faint yellow granules seen in unstained preparations. The outgrowths were more abundant in the adrenal than in the tumor explants. During 2 months cultivation the cells retained their morphological characteristics, although they became smaller. Two subcultures in series were obtained from the adrenal but none from tumor explants. After 7 weeks most cultures deteriorated and were discarded. The catecholamines were estimated by the method of v. Euler and Lishajko(2). One or 2 ml of culture medium was diluted to 25 ml with distilled water and treated as 25 ml urine in the above method. Catecholamines in the medium of first patient's tumor culture were determined on third day only. The culture medium was darkly pigmented and contained 0.56 μg NA/ml and 0.26 μg A/ml. In the second case of pheochromocytoma the catecholamines were determined for 2 parallel groups of tumor and adrenal explants. The media from 3 tubes were pooled first daily and later over periods of several days. Every tube contained 5-10 pieces (about 3 mm$^3$ each) of tissue and their outgrowths of varying size.

*Results.* Representative values of the catecholamine content of the media are given in Table I. The media from adrenal medulla cultures never contained detectable amounts of NA. The media from tumor cultures contained large amounts of NA on the first and smaller amounts on following 3 days. Catecholamines were found in fluids from both tumor and medulla cultures for at least 40 days. Experiments in which mixtures of small amounts ($<0.2$ μg/ml) of NA and A were estimated showed that by the method used the differentiation between NA and A is

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* Supported by Research Grants from Am. Cancer Scc. and Virginia Heart Assn.

TABLE I. Catecholamine Content in Culture Media of Pheochromocytoma and of Adrenal Medulla.

| | Pheochromocytoma | | | | Adrenal medulla | | | |
|---|---|---|---|---|---|---|---|---|
| Days after expl. | NA, $\mu$g/ml | | A, $\mu$g/ml* | | NA, $\mu$g/ml | | A, $\mu$g/ml* | |
| | I | II | I | II | I | II | I | II |
| 1 | 1.88 | 2.2 | 1.34 | 7.75 | 0 | 0 | .075 | .075 |
| 2 | .50 | 0 | 1.70 | .75 | | | | |
| 4 | .125 | .25 | .55 | 1.00 | 0 | 0 | .15 | .09 |
| 6 | 0 | 0 | .75 | .45 | | | | |
| 7 | 0 | 0 | .15 | .30 | | | | |
| 9 | 0 | 0 | .30 | .30 | | | | |
| 15 | 0 | 0 | .08 | .15 | 0 | 0 | .08 | .15 |
| 20 | 0 | 0 | .15 | .15 | 0 | 0 | 0 | .30 |
| 41 | 0 | 0 | .15 | .15 | | 0 | | .30 |
| 62 | 0 | | .037 | | 0 | | 0 | |

* The method of analysis as used did not reliably separate NA and A in quantities smaller than 0.02 $\mu$g/ml.

not quite reliable and all catecholamines tend to appear as A. Failure to find NA in culture fluids should not necessarily be attributed to the method used, since it was shown previously(3) that in adrenal glands of adult humans the largest portion of catecholamine is A.

*Summary.* Human adrenal medullary tumors and adrenal medulla produced catecholamines in tissue culture for at least 40 days.

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# Continuous Cultivation of Cells Arising from Human Case of Glioblastoma Multiforme (Glioma T.C. 178).* (25365)

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A variety of normal and malignant cells has been successfully cultivated *in vitro* and propagated in continuous subcultivations. There is, however, no report about continuous subcultures of cells arising from a glioma, although neoplasms of the nervous system have been cultivated for limited periods of time (1-6). In this communication we describe a line of cells derived from a human case of glioblastoma multiforme. These cells have been growing in continuous subcultures in this laboratory since Sept. 4, 1957.

*Materials and methods.* Fresh tumor tissue (5x5 mm) was immediately obtained from the operating room during a right temporal craniotomy for what was later histologically diagnosed as a glioblastoma multiforme of the right frontal lobe. The tumor was minced with uterine scissors and washed several times with balanced salt solution until supernatant fluid was clear. The small quantity of tumor fragments was evenly divided and distributed into three 8 oz. wide-mouthed Blake bottles. Approximately 20 cc of medium (composed of Eagles medium,† glutamine† 1%, Penicillin 100 units/cc, and Streptomycin 100 $\mu$g/cc), containing 30% human serum, was added to each bottle. During cultivation of cells, constituents of medium were kept constant in amount, the only variation being source and quantity of serum. The bottles were placed in 37°C incubator. On second day growth was observed around the explants. This continued slowly until 3 weeks later when all bottles showed luxuriant growth; the glass surface was not entirely covered with cells. At that time the original medium and floating necrotic fragments were discarded and 20 cc of new medium containing 20% human serum and 5% new-born human brain cell-free extract was added; Mycostatin (100 units/cc) was also added to eliminate the possibility of fungal growth. When surface of the Blake bottle was covered with cells, the culture was scraped with a rubber policeman, the cells centrifuged at 500 rpm for 5-10 minutes, resuspended in new medium, and planted in new bottles. The

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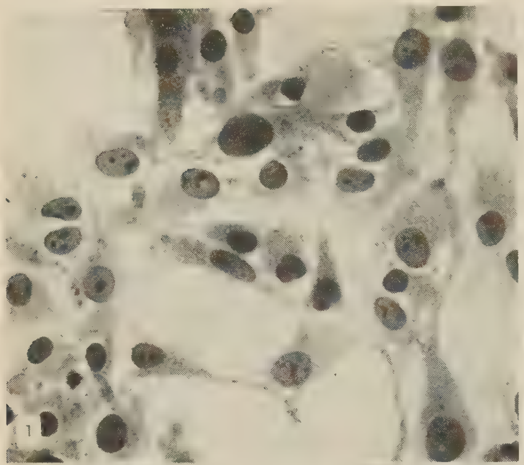
* Supported by U.S.P.H.S. Grant.

† Available from Microbiological Assoc., Washington, D.C.



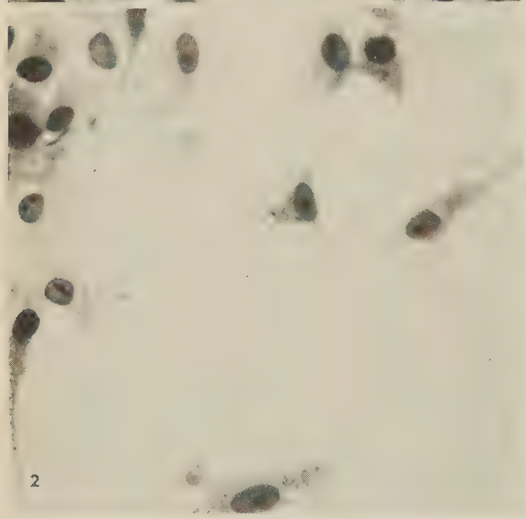


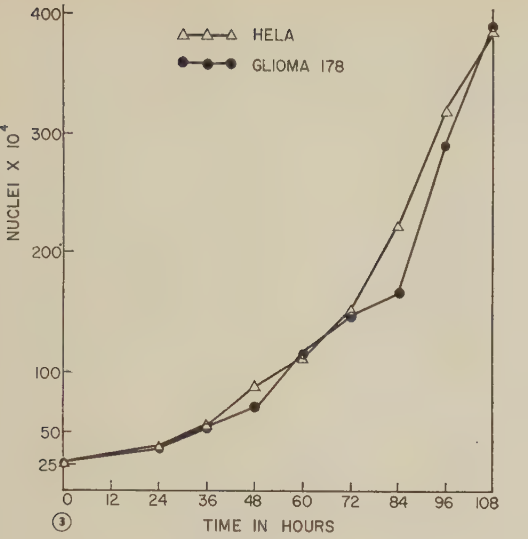
FIG. 1. Round, oval, and elongated nuclei are seen with varying numbers of nucleoli-like chromatin condensations. Hematoxylin and Eosin stain × 690.

FIG. 2. In the center of this picture cells are seen with irregular cytoplasmic processes. Hematoxylin and Eosin stain × 690.

cells were transferred in this manner from bottle to bottle 7 times in 20% human serum for 4 months. For the next 3 months growth was adequate using medium containing 10% human serum. Seven months from date of growth in tissue culture and with excellent growth in 10% human serum, growth was attempted in 10% horse serum. At first the cells grew slowly and much debris was noted in the bottles. Gradually the cells grew in horse serum and have been maintained in that medium to the present. For histological examinations cells were grown on coverslips in Leighton flat tubes. Coverslips were fixed in a variety of fixatives, dehydrated, and covered with a thin layer of paraffin. Using this technic, one may fix large numbers of coverslips and stain them at one's convenience according to the desired histological or histochemical technics. *Histology*. Tissue culture cells revealed pleomorphism; elongated, triangular, and pyriform cells were observed. Some cells were extremely large. Occasional multi-nucleated giant cells were seen. Size of nuclei varied considerably; some were very large; some oval, others round or often elongated. Variable numbers, often up to 7, of nucleoli-like condensations of chromatin were seen. Cytoplasmic details were to some extent obscured in regions of intense growth (Fig. 1). In periphery of culture and in other regions where growth was not so dense, the cells had elongated cytoplasmic processes (Fig. 2) that arose, in many instances, from one part of the cytoplasm and then bifurcated to form 2 unequal branches. Some cells had more than one cytoplasmic process. Often the cytoplasmic process of one cell touched the cytoplasmic process of adjacent cell. In some instances there were cytoplasmic bridge-like structures between neighboring cells. Very often the cytoplasmic processes revealed irregularities and varicosities; part of the process next to cell body was narrow and became enlarged in the periphery. The processes of cells stained with silver when impregnated with silver carbonate.

*Results*. Growth rate of glioma cells was estimated by nuclei counts and total protein determinations as follows: From healthy cultures in log phase of growth, 64, 3 oz. Brockway bottles were prepared each containing approximately 250,000 cells suspended in 10 cc of medium with 10% horse serum. Beginning with 24th hour, and every 12 hours thereafter to the 108th hour, 4 bottles were taken for nuclei counts and 4 bottles were simultaneously taken for total protein determinations. Average total nuclei count/bottle was determined by Rappaport's modification of the method of Parker *et al.*(7). Average amount of total protein/bottle was determined by the method of Oyama and Eagle(8).

Using exactly the same technics and me-



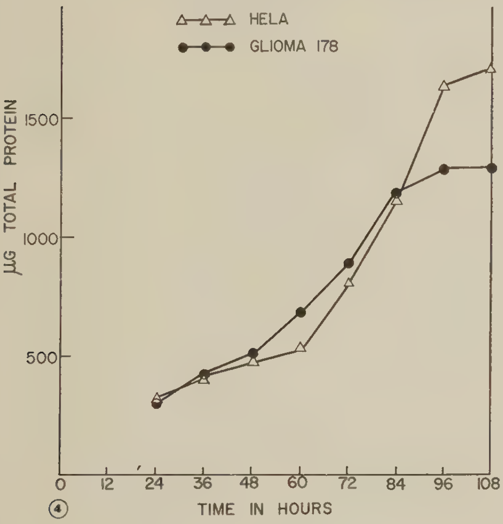


FIG. 3. Avg total nuclei count/bottle ($\times 10^4$) is given for Glioma T.C. 178 cells and HeLa (S-3) cells grown under identical environmental conditions.

FIG. 4. Avg total protein, µg/bottle, is given for Glioma T.C. 178 cells and HeLa (S-3) cells grown under identical environmental conditions.

dium as that previously described above for the glioma cells, rate of growth was determined for HeLa cells (Strain S-3). The comparative results of nuclei counts of Glioma T.C. 178 and HeLa cells are given in Fig. 3 and total protein determination for both is given in Fig. 4. From these graphs it is evident that rate of growth, in log phase under identical nutritional conditions, is approximately the same for Glioma T.C. 178 cells and HeLa cells; generation time for each is approximately 26 hours.

Preliminary experiments indicate that both West Nile and Poliomyelitis viruses may be propagated in Glioma T.C. 178 cells. Cells of Glioma T.C. 178, injected subcutaneously into the flank of X-irradiated cortisone-treated hamsters according to method of Toolan(9), cause development of small tumors at site of injection. These observations and results of heterologous transplantation of the glioma cells into guinea pig brains will be reported.

*Summary.* A line of tissue culture cells originally arising from a histologically verified human case of glioblastoma multiforme has been propagated in continuous subcultures for approximately 2 years. Morphological characteristics and preliminary observations concerning susceptibility of these cells to viruses are given. Rate of growth was measured by nuclei count and total protein determination and was compared with rate of growth of HeLa cells under identical conditions.

The authors express appreciation to Drs. F. Robinson and D. Cavicke for supplying the original tumor fragment and to E. Mullaly and G. Morann for technical assistance.

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# Kidney as a Source of Blood Ammonia: Effect of Chlorothiazide.* (25366)

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Elevated blood ammonia values have been observed following oral administration of chlorothiazide to patients with liver disease (1,2). However, the source of this increment of blood ammonia has not been identified. Recent studies indicate that the kidney is largely responsible for hyperammonemia which follows intravenous administration of the potent carbonic anhydrase inhibitor, Diamox®(3). Since chlorothiazide also increases urine pH and decreases urine ammonia excretion through carbonic anhydrase inhibition(4), the present study was undertaken to determine the effect of intravenous chlorothiazide on arterial ammonia concentration and release of ammonia into the renal vein.

*Methods.* Seven patients with Laennec's cirrhosis were studied. All were hospitalized males ranging in age from 28 to 49 years. None of the patients exhibited neuropsychiatric abnormalities or evidence of progressive hepatic deterioration. Renal function and serum electrolytes were normal. Patients were studied in recumbent position after overnight fast. A constant intravenous infusion (Bowman pump), delivering 14 to 16 mg of para-amino hippurate (PAH)/min. was maintained throughout and was preceded by a priming dose of PAH calculated to provide plasma levels of about 2 mg/100 ml. Each subject ingested 1000 ml of water at onset of study to initiate water diuresis. This was subsequently maintained by intravenous infusion of 5% Dextrose and water at 15 to 20 ml/min. Arterial and renal-venous blood was obtained from a brachial artery and a catheterized right renal vein. Control sampling was followed by intravenous administration of 500 mg of chlorothiazide. Subsequent samples were withdrawn at 10, 20, 30, 45, and 60 min. Voided urine specimens were obtained whenever possible. Arterial and renal-venous blood were analyzed for ammonia ($NH_3$), PAH, pH, $CO_2$ content, and $O_2$ saturation by previously described methods(3,5,6). Renal blood flow (RBF) was calculated from the arterial-renal venous (A-RV) PAH difference, using the indirect Fick principle of Bradley (7). The A-RV ammonia difference and RBF were used to calculate renal vein ammonia release ($\mu$g/min)(3).

*Results.* The effect of intravenous chlorothiazide on arterial ammonia concentration, renal vein ammonia release, and urine pH are shown in Fig. 1.

Mean arterial ammonia concentration progressively increased following chlorothiazide and was significant ($p = <0.01$) at 45 and 60 min. periods when compared with resting values. Signs of early pre-coma appeared in one subject, whose arterial ammonia concentration increased from 165 to 194 $\mu$g%.

The resting A-RV ammonia difference was negative in each subject, mean = –37 $\mu$g%, indicating release of ammonia into renal vein. Ten min. after chlorothiazide administration, mean renal vein ammonia release increased from control value of 1154 $\mu$g/min. to 1992 $\mu$g/min., $p = <0.01$, and remained essentially unchanged throughout 60 min. observation. No alterations in renal blood flow were observed, mean control = 1475 ml/min., 10 min. = 1571 ml/min., 20 min. = 1688 ml/min., 30 min. = 1444 ml/min., 45 min. = 1460 ml/min., and 60 min. = 1430 ml/min. Increase in quantity of ammonia released into the renal vein was associated with rapid and significant increase in mean urine pH, $p = <0.05$ and $<0.01$ at 10 and 60 min., respectively.

Arterial and renal-venous pH, $CO_2$ content and the calculated $pCO_2$ remained essentially unchanged throughout the study, as did A-RV oxygen differences and calculated renal oxygen consumption.

*Discussion.* The present data demonstrate that intravenous administration of chlorothiazide to patients with liver disease with or with-

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* Work supported (in part) by U. S. Public Health Grant.

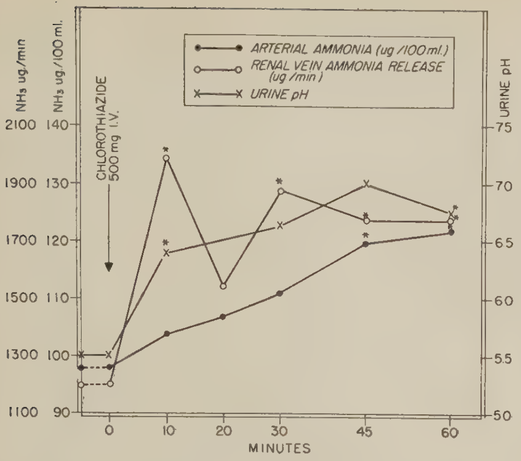

FIG. 1. Arterial ammonia concentration, release of ammonia into renal vein and urine pH before and after intrav. chlorothiazide. Mean values in 7 cirrhotic patients. * denotes significance.

out hyperammonemia results in a rise in arterial ammonia concentration, and that this rise is accompanied by an increased renal release of ammonia into the circulation. Indeed, increased release of ammonia into the renal vein could theoretically account for 360 $\mu g\%$ rise in extracellular fluid ammonia concentration at the 60 min. period, if one assumes a uniform distribution into a 14 liter fluid volume and an unchanged tissue uptake. Since the observed increase in arterial ammonia concentration was 22 $\mu g\%$, it seems likely that the kidney represents the major source of increased increment of blood ammonia which followed intravenous chlorothiazide. The present results parallel those previously reported when Diamox® was given to patients with liver disease(3). In addition, the magnitude of increased release of ammonia into the renal vein which followed Diamox® could be correlated with degree of increase in urine pH and decrease in urine ammonia excretion. Thus, the post-Diamox® data suggested that urine pH is not only an important determinant of urinary ammonia excretion but also influences the amount of ammonia liberated into the renal vein(3). In this study, urine pH promptly rose after I.V. chlorothiazide in association with the increase in release of ammonia into the renal vein. These observations lend additional support to the concept that the kidney is a significant source of blood ammonia and that factors which influence urine pH also influence this contribution.

*Summary*. The effect of intravenous chlorothiazide on arterial ammonia concentration and release of ammonia into renal vein was studied in 7 patients with Laennec's cirrhosis. Rapid and significant increases in quantity of ammonia released into the renal vein were observed along with progressive rise in arterial ammonia concentration. The data demonstrate the kidney's importance in the pathogenesis of hyperammonemia which follows intravenous chlorothiazide.

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# Effect of Lysergic Acid Diethylamide and Bufotenine on Performance of Trained Rats. (25367)

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The psychogenic activity of lysergic acid diethylamide (LSD) has long been recognized. It has been reported that a condition resembling schizophrenia can be produced in humans by administration of only 1 $\mu g$ of LSD/kg(1). Winter and Flataker(2) demonstrated that the effects of LSD upon trained rats bear a linear relationship to the log dose of the drug. These authors also reported a prolongation of climbing time of trained rats following injections of plasma from psychotic patients(3). Various other substances such as harmine, yohimbine and mescaline(4,5), have been used to produce abnormal mental conditions. Fabing and Hawkins(6) reported bufotenine to cause visual hallucinations in man and a bizarre syndrome of activity in rats and dogs. Since the endogenous production of bufotenine in humans remains a possibility(7), it was considered important to examine the psychogenic activity of bufotenine more closely. In the present report a comparison was made between psychogenic effects produced in trained rats by administration of LSD and bufotenine.

*Methods and materials.* The technic used for evaluating alterations in mental activity after administration of psychogenic drugs was similar to that described by Winter and Flataker(2). It consisted of measuring the delay in climbing time of trained rats; which in the present study were trained to climb a 5 foot, vertical, cloth covered pole rather than a rope. Rats of either sex weighing 100-250 g were used. Since fasting the animals did not appear to influence the outcome of these studies, they received food and water *ad lib.* All drugs were injected intraperitoneally (I.P.). Solutions were made fresh as needed. In those experiments where attempts were made to alter the effect of LSD or bufotenine, the animals were pretreated with reserpine,* 5-hydroxytryptophan (5-HTP) and iproniazid* (1-isonicotinyl-2-isopropylhydrazine) at 30, 90 and 180 minutes respectively. Injections of iproniazid are expressed as its phosphate salt, bufotenine as its mono-oxalate $H_2O$ complex and 5-hydroxytryptophan as the free amino acid; reserpine as its phosphate salt.

*Results. Effect of LSD and bufotenine on trained rats*: Both LSD and bufotenine caused prolongation of climbing time of trained rats. The quantitative aspects of this effect are shown in Fig. 1. Here total seconds prolongation is plotted against amount of drug injected. In Table I results are recorded in terms of C.T.D. (climbing time delay). Expressed either way the results show a direct proportionality between amount of drug administered and its effect on behavior of these rats.

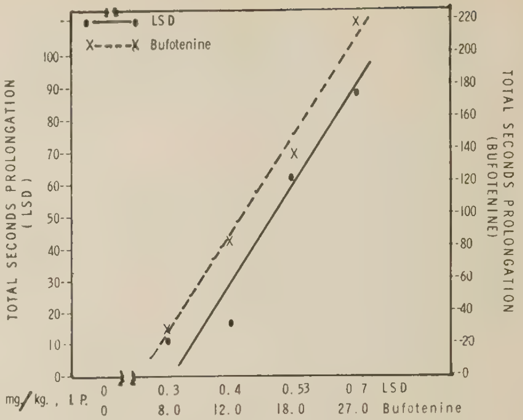

FIG. 1. Total seconds prolongation of trained rats relative to log dose of LSD and bufotenine.

There are differenecs as well as similarities in the effects of LSD and bufotenine intoxication. With either drug the action becomes apparent within 5-10 minutes after administration. However, with LSD the effect of the drug wears off in about 20 minutes. With bufotenine, on the other hand, intoxication

TABLE I. Climbing Time Delay for 5 Trained Rats per Dose after Psychogenic Drug Administration.

| Drug | mg/kg IP | C.T.D.† | Observation, min. |
|---|---|---|---|
| 1. LSD | .0* | .0 | 30 |
| | .3 | 58.5 | ” |
| | .4 | 101.0 | ” |
| | .53 | 383.5 | ” |
| | .70 | 426.5 | ” |
| 2. Bufotenine | .0* | 124.0 | 120 |
| | 8.0 | 330.0 | ” |
| | 12.0 | 746.5 | ” |
| | 18.0 | 1667.5 | ” |
| | 27.0 | 2397.5 | ” |
| 3. (Bufotenine tolerant rats) | | | |
| LSD | .53 | .0 | 30 |
| 4. (LSD tolerant rats) | | | |
| Bufotenine | 27.0 | 26.5 | 60 |

* Saline inj.

† C.T.D. value takes into consideration the intensity and duration of prolongation in climbing time; computed according to Winter and Flataker(2).

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* Reserpine used was donated by Ciba Pharmaceutical Products, Summit, N. J., Iproniazid donated by Hoffmann-La Roche, Nutley, N. J.

may last as long as 90-120 minutes. Thus while both LSD and bufotenine affect behavioral characteristics of the test animal, LSD produces its effect at a much lower dose than does bufotenine. The confused state brought about by either drug often caused animals to climb only part way up and return to the stimulating grid instead of making a systematic ascent to the platform. This striking phenomenon is difficult to quantitate. Administration of either LSD or bufotenine produced a peripheral vasodilation as evidenced by bright red color of ears and paws. The effects of the 2 drugs differed in that LSD produced hypersalivation and increased frequency of defecation and urination, whereas these effects were not observed following bufotenine injection. This phase of the study substantiates results of Winter and Flataker(2) with respect to LSD. However, the effect of administered psychotic plasma to such animals, as observed by these authors(3), could not be confirmed.

*Tolerance to bufotenine and LSD*: A tolerance to the behavioral and pyretogenic responses to LSD has been reported(8). During our study, after 4 or 5 injections of bufotenine the rats not only became refractory to further injections of this drug, but developed a tolerance to LSD as well (Table I-3). Similarly, rats made refractory by several injections of LSD became tolerant to both LSD and bufotenine (Table I-4).

TABLE II. Alteration of Climbing Time Delay of LSD and Bufotenine Treated Rats.

| Drug, mg/kg, IP | | No. of animals per dose | C.T.D. | Avg duration of drug action, min. |
|---|---|---|---|---|
| 1. Reserpine | LSD | | | |
| | .3 | 1 | | 60 |
| 1.25 | .3 | 1 | 326 | ” |
| 1.25 | | 2 | 62 | ” |
| 2. | Bufotenine | | | |
| | 8 | 2 | 818 | 120 |
| 5 | 8 | 2 | 6780 | ” |
| 5 | | 2 | 994.5 | ” |
| 3. 5-HTP | LSD | | | |
| | .5 | 5 | 943.9 | 40 |
| 100 | .5 | 5 | 443.5 | ” |
| 4. | Bufotenine | | | |
| | 27 | 4 | 2467 | 60 |
| 100 | 27 | 4 | 1483 | ” |
| 5. | LSD | | | |
| | .3 | 5 | 77.5 | 40 |
| 100 | .3 | 5 | 173.3 | ” |
| 6. | Bufotenine | | | |
| | 12 | 5 | 450.5 | 60 |
| 100 | 12 | 5 | 842.7 | ” |
| 7. Iproniazid | LSD | | | |
| | .3 | 3 | | 30 |
| 120 | .3 | 4 | 172.7 | ” |
| 8. | .5 | 5 | 252.7 | ” |
| 120 | .5 | 6 | 1344 | ” |
| 9. | Bufotenine | | | |
| | 12 | 5 | 26.3 | 60 |
| 120 | 12 | 6 | 1186 | ” |

*Potentiation of LSD and bufotenine effect by reserpine*: Winter and Flataker(2) reported a potentiation of LSD effect by administration of reserpine. These findings could be confirmed in our study, although only a small number of animals were used. When reserpine (1.25 mg/kg) was administered along with LSD (0.3 mg/kg) the effect was greater than that of LSD or reserpine alone (Table II-1). A similar effect on the bufotenine action could also be demonstrated (Table II-2). Thus when reserpine (5 mg/kg) was given along with a minimally effective dose of bufotenine (8 mg/kg) the C.T.D. was increased several fold as compared to that obtained when a similar amount of bufotenine was injected alone. Since, as already mentioned, the rats developed a tolerance to repeated injection of the 2 psychogens, a new group of rats had to be used for each study. Any variations occurring as a result of this procedure were controlled by following a cross-over pattern of injection.

*Alterations in psychogenic activity of drugs as a function of brain serotonin levels.* Experiments were carried out to illuminate the possible effect of increased brain levels of serotonin on the action of the psychogenic drugs. Animals were pretreated with 5-hydroxytryptophan (5-HTP) or with iproniazid before receiving the psychogenic drugs. Ample evidence has been reported that administration of either 5-HTP or iproniazid(9) will raise the brain level of serotonin (5-HT), hence no actual determinations of brain 5-HT were carried out. Injection of trained rats with 100 mg/kg 5-HTP produced no detectable change in behavior of these animals. However, when such administration of 5-HTP was followed 90 minutes later by an injection of 0.5 mg/kg LSD, the usual response to such a dose of LSD was reduced considerably (Table II-3). But when a group of similarly 5-HTP pretreated rats were injected with only 0.3 mg/kg LSD a prolongation rather than a reduction of C.T.D. was observed (Table II-5). These experiments were repeated substituting bufotenine for LSD. When rats pretreated with 100 mg/kg 5-HTP were injected with a relatively large dose of bufotenine a reduction in C.T.D. was produced (Table II-4). If the 5-HTP pretreated rats were injected with a smaller dose of bufotenine (Table II-6) the C.T.D. was increased.

Another method for increasing the level of serotonin in the brain relies on inhibition of monoamine oxidase system by iproniazid. One would expect, therefore, that pretreatment of rats with iproniazid prior to injection of LSD or bufotenine should produce effects similar to those seen after pretreatment with 5-HTP. As Table II shows this is actually the case. Pretreatment with iproniazid increased the C.T.D. in every case with either LSD or bufotenine. Here again similarity in action for these 2 psychotropic drugs is demonstrated.

*Discussion.* The method of Winter and Flataker(2) was used in our study, because it has features making it particularly suitable for this type of work. Thus it is neither difficult nor tedious to train rats to climb a pole in a reproducible length of time. This was of particular value, since the animals quickly developed a tolerance and cross-tolerance for the 2 drugs (LSD and bufotenine), hence they could be used only for a limited number of trials. The disadvantages of the pole climbing technic for assaying psychogenic drugs are also quite apparent. Ability of a rat to climb a 5 foot pole in a reasonable length of time depends upon 2 factors: (a) a mental factor, the will to climb, and (b) a muscular factor, the strength necessary to do so. Hence any results obtained by this method should be interpreted with some caution.

That these psychogenic drugs produce their effect by interfering with normal serotonin metabolism seems particularly attractive(10). In the present study the effects of 2 serotonin analogs, LSD and bufotenine were compared. Both of them are capable of producing confusion and behavioral changes in trained rats. Upon repeated injections of each of these 2 drugs the rats readily developed a tolerance to them. Even more interesting is the observation that rats made refractory to one of these drugs also became tolerant to the other. This induced cross-tolerance strongly suggests that both LSD and bufotenine produce their psychogenic effect by the same mechanism. Any treatment known to affect serotonin metabolism and which can be shown to modify the action of both LSD and bufotenine would lend support to this concept.

Reserpine has definite tranquilizing properties and also causes a release of bound serotonin in the brain and other tissues(11). Hence increased C.T.D. shown in Table II when reserpine was administered along with either LSD or bufotenine could well be related to its interference with bound serotonin level in the brain.

More clear cut results were obtained when procedures known to increase the level of brain serotonin were employed. Iproniazid has been shown to block the monoamine oxidase system, which probably is the main pathway for normal catabolism of serotonin. Prior administration of iproniazid greatly potentiates the action of both LSD and bufotenine (Table II). Similarly pretreatment of rats with 5-hydroxytryptophan potentiates the effects of LSD and bufotenine when administered in doses of 0.3 and 12 mg/kg respectively. However, an anomaly occurred with both LSD and bufotenine. With prior administration of 100 mg/kg 5-HTP the effect of small doses of both psychogenic drugs was enhanced. The effect of larger doses of drugs on the other hand was reduced by prior administration of 5-HTP. Further studies are required to illuminate this finding. Yet the interesting feature is that the result of 5-HTP pretreatment modifies the effect of LSD and bufotenine in a similar manner, thus strengthening the hypothesis that these 2 drugs produce their psychogenic action through the same mechanism or target cells.

*Summary.* 1. A comparative study has

been carried out to determine the effect of LSD and bufotenine on climbing time of trained rats. 2. Both drugs produced similar effects in these animals. However, LSD was effective at smaller doses but had a shorter duration of action than bufotenine. 3. Tolerance to repeated LSD injections was found and a similar tolerance to bufotenine was demonstrated. 4. Prolongation of climbing produced by submaximal doses of both LSD and bufotenine was potentiated when brain serotonin levels were altered by pretreatment of animals with reserpine, 5-HTP or iproniazid. Highly effective doses of LSD and bufotenine were inhibited by 5-HTP pretreatment. 5. Based on evaluation of climbing time delay produced in trained rats the psychogenic activity of LSD and bufotenine appears to depend on a similar mechanism of action.

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## Effect of Glucagon on Renal Functions in the Dog.* (25368)

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Glucagon, in addition to effects on carbohydrate metabolism(1) has been reported to increase urinary excretion of electrolytes in dog (2) and in man(3,4). These authors agree that the electrolyte effects of glucagon are independent of its hyperglycemic action. They also feel that the effects of glucagon on electrolyte excretion probably result from a direct action on the renal tubule since they observed only occasional or slight increases of equivocal significance in glomerular filtration rate (GFR) and renal plasma flow (RPF). Glucagon does not alter peripheral blood flow as measured by finger plethysmography(5), but does produce a marked increase in hepatic blood flow(6). In view of the equivocal data available regarding glomerular filtration rate and renal plasma flow after glucagon administration, and since small changes in glomerular filtration rate could produce large changes in electrolyte excretion, this problem has been further investigated in the dog. In addition, the effect of glucagon on maximum rate at which the renal tubules can reabsorb glucose (Tm glucose) was investigated. In a recent study(7), glucagon was found to have no effect on $Tm_G$ in 2 volunteer subjects.

*Materials and methods.* Seven healthy trained unanesthetized female mongrel dogs between 45 and 55 lb in weight were used in this study. Exogenous creatinine clearance (GFR), p-amino hippurate (PAH) clearance (RPF) and Tmglucose ($Tm_G$) were measured by standard technics(8,9). Briefly, creatinine and PAH or creatinine and glucose were administered at a constant rate by means of an infusion pump. After a suitable period allowed for equilibrium, urine was collected by catheter. Each collection period was terminated by a bladder rinse with distilled water. Urine collection periods were accurately timed. Blood was obtained at mid-

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TABLE I. Effect of Glucagon (HGF) on Glomerular Filtration Rate (GFR), Renal Plasma Flow (RPF) and Filtration Fraction (FF) in Dog.

| Function | Procedure | No. dogs | No. exp. | Renal function |  |  | P (paired comparison of basal *vs* exp. groups) |
|---|---|---|---|---|---|---|---|
|  |  |  |  | Basal | Exp. | Δ |  |
| GFR (ml/min.) | Control infusions | 5 | 9 | 66.2 | 69.3 | + 3.1 | >.1 |
|  | Glucagon | 7 | 26 | 59.9 | 75.2 | +15.3 | <.001 |
| RPF ” | Control infusions | 3 | 4 | 230 | 282 | +52 |  |
|  | Glucagon | 3 | 14 | 190 | 256 | +66 | <.001 |
| FF (%) | Control infusions | 3 | 4 | 32.5 | 29.6 | − 2.9 |  |
|  | Glucagon | 3 | 14 | 28.7 | 28.9 | + .2 | >.5 |

point of each collection period. Arterial plasma glucose was measured when $Tm_G$ was to be calculated. After appropriate dilutions plasma and urine were analyzed for PAH(9), creatinine(10) and glucose(11). The plasma filtrates were treated with Folin decalso and Lloyd reagent to remove creatinine prior to the glucose analysis. Glucose in the concentration range achieved in our experiments did not alter creatinine values.

Following 3 control periods, crystalline insulin-free glucagon[†] (HGF) was administered by single injection intravenously or intraperitoneally, or by continuous intravenous infusion at a measured constant rate by means of an infusion pump. Vehicles for HGF included 0.85% saline, glycine-NaOH buffer (pH 8.5) and dog plasma. Renal functions were measured for 3 or more 10 to 20 minute periods during the continuous infusion of HGF at 6 to 45 $\mu$ per minute, or for 15 to 30 minute periods after the single intraperitoneal or intravenous injection of 0.2 to 0.5 mg HGF. Frequent measurements of rectal temperature and mean arterial blood pressure were made during 5 experiments. No significant changes were observed. As controls, similar measurements of renal functions were made before and during continuous intravenous administration of glycine-buffer, saline or glucose in amounts used for the $Tm_G$ measurements. As a further control, renal functions were similarly measured when HGF which had been heated to 100°C for 1 or 12 hours in buffered solution at pH = 7.04 was administered by continuous intravenous infusion in 3 experiments.

In long term studies, HGF in saline was given intraperitoneally, 0.5 mg once daily for 145 days in Dog 1, and 0.5 mg twice daily for 140 days in Dog 2. Renal functions were measured at intervals throughout the experimental periods. Urine was tested for sugar frequently. At the end of experimental periods percutaneous renal biopsies revealed normal kidneys in both dogs.

*Results.* The effects of glucagon (HGF) on glomerular filtration rate (GFR), renal plasma flow (RPF) and filtration fraction (FF) are summarized in Table I. GFR and RPF increased after or during HGF administration in every experiment. Mean increases were statistically significant ($p<0.001$). Although GFR and RPF usually increased progressively after HGF, the 3 to 5 post-HGF periods were averaged and compared to the 3 pre-HGF control periods. HGF had no consistent effect on the FF ($p>0.5$). Route of administration, the vehicle, and rate of infusion of HGF within the limits tested did not appear to affect the results. All types of experiments, therefore, were considered to be a single group. Arterial plasma glucose invariably increased after HGF. In some experiments, however, plasma glucose increase was not sufficient to result in glycosuria. GFR increased as much in these experiments as in those in which glycosuria developed. Likewise, HGF increased GFR in those experiments in which marked glycosuria was already present due to glucose infusion. In control studies neither glucose nor saline infusions at 8 ml/min. affected GFR significantly ($p>0.1$). RPF did increase in each of 4 control experiments but the data were too few to establish statistical significance.

The effects of HGF on $Tm_G$ were studied in 11 experiments in 7 dogs. In all experiments

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[†] Lot No. 258-234B-54-2, supplied through courtesy of Dr. W. R. Kirtley, Eli Lilly Research Laboratories, Indianapolis, Ind.

TABLE II. Effect of Daily Administration of Glucagon (HGF) on Renal Functions in 2 Dogs.

| Dog No. | Wk of HGF | GFR (ml/min.) | RPF (ml/min.) | FF (%) | $Tm_G$ (mg/min.) | $Tm_G$/GFR |
|---|---|---|---|---|---|---|
| 1 | 0 | 62.4* | 235† | 29.9† | 284 | 4.8 |
| | 6 | 74.7 | | | 470 | 6.3 |
| | 10 | 55.6 | 184 | 30.2 | 274 | 4.7 |
| | 12 | 59.8 | | | 258 | 4.3 |
| | 16 | 56.6 | | | 258 | 4.0 |
| | 17 | 52.2 | 155 | 33.7 | | |
| 2 | 0 | 69.3† | 190 | 39.4 | 162 | 2.5 |
| | 8 | 64.3 | | | 261 | 4.1 |
| | 10 | 62.2 | | | 243 | 3.9 |
| | 11 | 71.1 | | | 310 | 4.5 |
| | 16 | 52.9 | 176 | 30.1 | | |

* Mean of 5 control studies. † Mean of 2 control studies. All other data represent one study.

plasma glucose levels were maintained sufficiently high to insure filtered glucose load to $Tm_G$ ratios greater than 1.5, which is generally accepted to be adequate(12). Again, GFR invariably increased after HGF (mean control GFR = 57.4 ml/min., mean GFR after HGF = 69.1 ml/min). Because the plasma glucose also invariably increased, glucose filtered at the glomerulus increased markedly after HGF. Despite this, mean $Tm_G$ increased in 4 experiments (4, 7, 29, and 42%), did not change in one, and decreased in 6 (17, 22, 24, 31, 33 and 37%). The effect of HGF on $Tm_G$ was not statisticaly significant ($p > 0.2$ by paired comparison). However, the $Tm_G$/GFR ratio decreased in 9 (9, 25, 24, 28, 30, 33, 44, 44 and 46%) and increased in only 2 experiments (7 and 21%). The mean decrease in the ratio after HGF was statistically significant ($p < 0.005$). In a single control experiment in which $Tm_G$ was measured for 8 fifteen minute periods, the mean $Tm_G$ for the first 4 periods was 162 mg/min. and for the last 4 periods was 211 mg/min. $Tm_G$/GFR ratios were 2.6 and 3.6 respectively.

In 2 dogs treated with daily doses of HGF for 140 and 145 days respectively, GFR, RPF, and $Tm_G$ did not change when measured 24 hours after a HGF dose (Table II). No tolerance to HGF developed, however, in that increased plasma glucose and GFR were observed after individual doses of HGF throughout the experiments.

Attempts to inactivate HGF by heating to 100°C for 1 or 12 hours failed, in that such preparations produced hyperglycemia and increased GFR in 3 experiments.

In a single experiment, HGF increased cardiac output (measured by the Fick principle) from 0.333 to 0.720 liter/min.

*Discussion.* HGF appears to increase GFR transiently but invariably in the dog. The increase is great enough to account for the increased electrolyte excretion observed by others(2), although other mechanisms of action of the hormone on renal electrolyte excretions are not excluded. We have no explanation for the failure of others to observe an increase in GFR after HGF, although variations in the preparation of HGF used perhaps might account for the difference (HGF lot numbers quoted by other investigators differed from ours). We are satisfied that various infusion rates, vehicles for HGF, hyperglycemia, glycosuria, or analytical errors do not account for the increased GFR observed in our experiments. HGF also increased RPF but did not significantly alter FF. The increased RPF after HGF is not surprising in view of the increased hepatic blood flow reported after this material(6) and the increased cardiac output we observed in a single experiment.

The effect of HGF on $Tm_G$ was not consistent nor statistically significant in 11 experiments. However, in 6 of the 11 experiments, $Tm_G$ decreased by more than 15% after HGF. Moreover, because of a consistent increase in GFR, $Tm_G$/GFR ratio was decreased significantly after HGF. Although by no means certain, these data are consistent with the possibility that the decreased $Tm_G$ and $Tm_G$/GFR ratio observed after insulin

(13) might in part have been due to contamination of the insulin with HGF.

*Summary.* HGF increases GFR and RPF consistently but transiently in the dog, does not affect FF, at times decreases $Tm_G$. Tolerance does not develop during long term daily HGF administration nor does this regimen increase the basal renal functions.

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## Electrophoretic Studies of Bovine Serum. IV. Differences in Serum Glycoproteins Due to Age and Sex.* (25369)

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Presence of protein-bound carbohydrates of the hexose variety in serum proteins has long been recognized, although very little is known concerning their physiological significance. Only recently has attention been focused on the clinical significance of these glycoproteins in human medicine(1,2) and to a limited extent in experimentation with small laboratory animals(3-5) and swine(6). It has been demonstrated that concentrations of serum glycoproteins are abnormally elevated in many pathological and physiological conditions(7-9) either as a result of tissue destructive(7,9) or proliferative processes(8,9). Limited data on glycoproteins in parasitic infestations in small laboratory animals have been published(10). In these cases significant alterations were not found. A study of serum glycoproteins of the bovine species has not been reported. Mention has been made that the albumin, alpha-, beta-, and gamma-globulins have some glycoprotein associated with each fraction as determined by paper electrophoresis(11). Due to our interest in various diseases of cattle, and the possible significance of serum glycoproteins in formulation of mechanisms of disease processes, an investigation was made of glycoproteins in normal bovine serum. This report presents information on the relative and absolute concentrations of glycoproteins associated with serum proteins. Values are also given with respect to age and sex differences.

*Materials and methods. Cattle*: Serum glycoproteins from 3 dairy herds of single and mixed breeds managed under southern Louisiana conditions were studied. Two herds consisted of 232 females ranging in age from 1 month to 13 years. The third herd consisted of 35 stud bulls ranging in age from 2 to 15 years. All animals were considered to be normal and appeared to be free from any apparent physiological or pathological disturbances. Blood was collected individually in early

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TABLE I. Statistical Summary of Relative Concentrations of Glycoproteins Bound to Components of Normal Bovine Serum Obtained from Various Age Groups.

| | | Mean % conc. and stand. dev. of glycoproteins bound to serum fractions | | | |
|---|---|---|---|---|---|
| | | | Globulins | | |
| Age group (mo) | No. of samples | Albumin | α | β | γ |
| >24 | 160 | 3.3 ± .3 | 50.0 ± .7 | 25.0 ± .5 | 21.7 ± .5 |
| 18–24 | 28 | 3.5 ± .6 | 53.0 ± 1.3 | 26.0 ± 1.5 | 17.6 ± 1.1 |
| 12–18 | 26 | 2.6 ± .8 | 55.4 ± 1.6 | 24.1 ± 1.1 | 17.9 ± 1.0 |
| 9–12 | 8 | 3.7 ± 1.5 | 62.5 ± 3.1 | 17.7 ± 3.6 | 16.1 ± 2.7 |
| 6– 9 | 14 | 6.4 ± 1.4 | 53.0 ± 2.6 | 20.9 ± 1.2 | 19.8 ± 2.4 |
| 3– 6 | 20 | 5.0 ± 1.3 | 58.0 ± 2.6 | 21.4 ± 1.2 | 15.6 ± 1.5 |
| <3 | 11 | 3.9 ± 1.2 | 63.4 ± 1.7 | 19.4 ± 1.0 | 13.4 ± 1.5 |
| F values* | | 1.95 | 9.12† | 5.60† | 7.11† |

* Analysis of variance. † Significant at .01 level of probability.

spring. Each serum sample was stored at −22°C until analyzed for glycoproteins, using paper electrophoretic technics and chemical determinations. Our unpublished data have shown that freezing and thawing 1 time does not alter glycoprotein content of bovine serum. *Electrophoresis*: Each serum sample was applied to a paper strip 4 times in 0.01 ml amounts giving total volume of 0.04 ml. This volume of serum was the minimum sufficient to yield measurable amounts of glycoprotein when using a photoelectric densitometer-integrator. Volumes above 0.04 ml produce smearing of proteins that are separated on paper and are not suitable. Proteins were separated in barbital buffer of pH 8.6 and 0.075 ionic strength. A current of 0.63 milliampere/strip was applied for 17 hours at 23°C. Then the strips were dried and fixed at 125°C for 30 minutes. *Staining and determination of glycoproteins*: All strips were stained with periodic acid Schiff reagent as described by Köiw and Grönwall(12). After drying at 23°C the strips were scanned for relative % composition of glycoproteins by using a densitometer-integrator equipped with 550 mμ interference filters. To convert these relative values of each glycoprotein associated with a specific serum fraction to absolute weights, it was necessary to determine total content of glycoproteins present. The tryptophan method of Shetlar, *et al.*(13) was used in analyses of duplicate samples of serum.

*Results.* Mean values and standard deviations of the relative concentrations of protein fractions are shown in Table I. Analysis of variance showed that the differences in relative amounts of albumin among 267 animals in 7 age groups were not statistically significant. It was evident that significant differences occurred in glycoproteins in that younger animals possessed relatively more alpha-globulin glycoprotein and less beta- and gamma-globulin glycoprotein than older animals.

Results obtained from a compilation of data on absolute concentrations of serum glycoproteins are presented in Table II. The data show that there were no significant differences in weights of glycoproteins associated with the

TABLE II. Statistical Summary of Absolute Concentrations of Glycoproteins Bound to Components of Normal Bovine Serum Obtained from Various Age Groups.

| | | Mean mg % conc. and stand. dev. of glycoproteins bound to serum fractions | | | | |
|---|---|---|---|---|---|---|
| | | | Globulins | | | |
| Age group (mo) | No. of samples | Albumin | α | β | γ | Total |
| >24 | 160 | 3.9 ± .4 | 60.7 ± 1.1 | 30.4 ± .6 | 26.3 ± .7 | 121.3 ± 1.3 |
| 18–24 | 28 | 3.7 ± .7 | 58.0 ± 2.0 | 28.3 ± 1.2 | 18.9 ± 1.1 | 108.9 ± 1.7 |
| 12–18 | 26 | 2.7 ± .8 | 62.5 ± 2.8 | 26.8 ± 1.3 | 20.0 ± 1.3 | 112.0 ± 2.8 |
| 9–12 | 8 | 3.7 ± 1.5 | 63.6 ± 3.0 | 18.4 ± 3.7 | 16.4 ± 2.7 | 102.2 ± 2.9 |
| 6– 9 | 14 | 6.9 ± 1.4 | 58.6 ± 3.7 | 23.2 ± 1.8 | 21.9 ± 2.1 | 110.6 ± 5.1 |
| 3– 6 | 20 | 4.9 ± 1.3 | 59.5 ± 3.2 | 21.8 ± 1.2 | 15.9 ± 1.5 | 102.0 ± 2.1 |
| <3 | 11 | 3.7 ± 1.1 | 58.9 ± 1.4 | 18.1 ± 1.1 | 12.5 ± 1.5 | 93.1 ± 1.5 |
| F values* | | 1.50 | .520 | 11.59† | 12.88† | 14.63† |

* Analysis of variance. † Significant at .01 level of probability.

TABLE III. Statistical Summary of Relative Concentrations of Glycoproteins Bound to Components of Normal Male and Female Bovine Serum from a Similar Age Group (2 Years and Older).

| Sex and No. of samples | | Mean % conc. and stand. dev. of glycoproteins bound to serum fractions | | | |
|---|---|---|---|---|---|
| | | | Globulins | | |
| | | Albumin | α | β | γ |
| ♂ | 35 | 3.7 ± .6 | 43.0 ± 1.0 | 30.2 ± 1.0 | 23.1 ± 1.0 |
| ♀ | 125 | 3.2 ± .4 | 51.9 ± .7 | 23.6 ± .4 | 21.2 ± .6 |
| F values* | | .137 | 36.83† | 48.53† | 1.73 |

* Analysis of variance.
† Significant at .01 level of probability.

TABLE IV. Statistical Summary of Absolute Concentrations of Glycoproteins Bound to Components of Normal Male and Female Bovine Serum from a Similar Age Group (2 Years and Older).

| | | Mean mg % conc. and stand. dev. of glycoproteins bound to serum fractions | | | | |
|---|---|---|---|---|---|---|
| | | | Globulins | | | |
| Sex | No. of samples | Albumin | α | β | γ | Total |
| ♂ | 35 | 4.6 ± .8 | 53.6 ± 1.5 | 37.7 ± 1.4 | 29.9 ± 1.5 | 124.8 ± 2.3 |
| ♀ | 125 | 3.8 ± .4 | 62.7 ± 1.2 | 28.3 ± .6 | 25.6 ± .8 | 120.4 ± 1.5 |
| F values* | | .794 | 13.27‡ | 49.09‡ | 4.31† | 2.35 |

* Analysis of variance. † Significant at .05 level of probability. ‡ Significant at .01 level of probability.

albumin and alpha-globulin fractions between age groups. It appeared that younger animals possessed significantly less total glycoprotein and beta- and gamma-globulin glycoprotein than older animals.

Analysis of variance between 35 males and 125 females, all 2 years of age and over, showed statistically significant differences in relative amounts of alpha- and beta-globulin glycoproteins. Table III indicates that males possessed less alpha-globulin glycoprotein and more beta-globulin glycoprotein than females. Significant sex differences did not occur in the other fractions.

Table IV presents data on sex differences in absolute weights of serum glycoproteins. Males possessed less alpha-globulin glycoprotein and more beta-globulin and gamma-globulin glycoproteins than females. There were no significant sex differences in weights of albumin glycoprotein and total glycoprotein.

*Discussion.* The reasons for differences in concentrations of glycoproteins associated with various serum fractions are not definitely known and have been the subject of speculation(9,14). Winzler(9) proposed that due to the multiplicity of serum glycoproteins it is possible that the different components are concerned with divergent physiologic processes. Possibly, these differences occur as a direct result of the maturation process of the calf into an adult when hormonal and other influences are in operation.

Reasons for lowered amounts of glycoproteins in serums of young animals are conjectural. Shetlar, *et al.*(14) reported that human fetal serum contains less polysaccharide than serum from adults. Younger bovine animals possess less total serum proteins(15) and possibly cannot bind as much glycoprotein.

The possibility that the amounts of serum proteins influence the amounts of glycoproteins bound, that the differences observed may occur as a result of lactation, hormonal influences between sexes, sexual maturity, metabolic rates, and other diverse physiological stimuli are subjects for further investigation.

*Summary.* Differences found in glycoproteins of bovine serum with respect to age and sex are presented.

The authors acknowledge the aid of Drs. B. R. Farthing in statistical analyses and J. L. Fletcher, R. B. Lank, and E. E. Roth for procurement of blood samples.

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# Effect of Sodium Chloride Concentration on Staphylococcal Agglutination.* (25370)

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Since it has been shown that concentration of salts affects direct bacterial agglutination (1,2), it was decided to evaluate the degree of this effect in macroscopic staphylococcal agglutination. Spontaneous agglutination, the quantity of agglutination as measured by highest agglutination titer and the quality of agglutination as determined both by reproducibility and sharpness of endpoint and gross particulation of aggregates were observed.

*Method and materials.* Four different strains of hemolytic coagulase positive staphylococci representing the following phage types were used†

| Strain | Phage type |
|---|---|
| a | 29 |
| b | 81 |
| c | 4, 6, 29, 29A, 31, 79, 44, 52, 52A, 71, 3A, 42E, 44A, 73, 75, 51, 81 |
| d | 51, 42B, 81 |

Antisera were obtained from rabbits immunized intravenously with these strains grown in broth and killed with 0.25% phenol. Human sera were obtained from adult male volunteers. The term agglutination reaction is restricted to visible (second) stage of agglutination and precipitation as determined macroscopically, and does not imply consideration of combination or dissociation of antibody and antigen. Antigen concentration was that giving a density equal to that of the Standard Typhoid Vaccine (1000 million organisms/ml) supplied by Division of Laboratories and Research, N. Y. State Dept. of Health. Agglutination reactions were carried out as follows: Serial 2-fold dilutions of serum were made in standard agglutination tubes (½ x 3 inch) with the desired concentration of salt solution. One half ml was left in each tube. The organisms were kept in frozen skim milk until used. The original or first subculture from skim milk was used as antigen. After overnight incubation on rabbit agar, the growth was removed with a sterile cotton swab from surface of plate and suspended in desired concentration of sodium chloride solution. After the suspension was filtered through a small cotton plug, it was adjusted to a density twice that of optimal antigen concentration. The antigen suspension was then added to the serial 2-fold dilutions of serum in a ratio of 1:1 thus giving final volume of one milliliter. The mixture was incubated in water bath at

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* This article expresses personal opinions of authors and does not necessarily reflect official opinion of Navy. Studies were aided by contract between Office of Naval Research, Dept. of Navy, and Univ. of Rochester, Rochester, N. Y.

† We are indebted to Dr. Louis J. Griffith, Veterans Hosp., Batavia N. Y., for typing these strains.

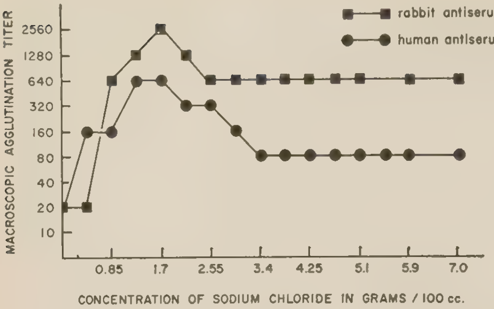

FIG. 1. Optimal concentration of NaCl for agglutination of hemolytic *Staphylococcus aureus* by antisera.

37° for one hour, placed in refrigerator overnight and observed the following morning for agglutination with the aid of a hand lens.

*Results. Optimal concentration of sodium chloride in staphylococcal agglutination.* Fig. 1 illustrates the macroscopic agglutination titer of a representative suspension of hemolytic *Staphylococcus aureus* by homologous immune rabbit serum and by human serum in various dilutions and at various concentrations of sodium chloride, the amount of antigen being constant. The highest serum titer in rabbit antiserum occurred at salt concentration of 1.7% sodium chloride. In human sera, again with constant amounts of antigen, highest serum titer was reached at salt concentration of from 1.3% to 1.7%. Agglutination titers in general were maximal at NaCl concentration between 1.3 and 2.1 and were significantly less below and above this range.

With human sera, there was improvement in quality of agglutination at 1.3% and 1.7% sodium chloride. Endpoints were sharper and were more easily reproduced. A tendency of aggregates to be ropy and difficult to read was observed at and below 0.85% sodium chloride. This did not occur at 1.3% or at greater concentrations of the salt. In rabbit antisera, marked improvement in quality of agglutination consistently occurred in concentrations of 3.2% sodium chloride and above. Spontaneous agglutination of control antigens was irregularly observed in saline concentrations of 0.85% and less.

These data show that variations in staphylococcal agglutination occur with changes in salt concentration as Duncan(1) found with *Bacterium dysenteriae,* but in different ranges of concentration.

*Solubility of antigen.* Solubility of agglutinating antigen of the staphylococcus is shown in Tables I and II. Organisms suspended in salt free solution, in 0.85%, in 1.3% and in 3.2% sodium chloride concentrations were immediately separated from the respective solutions by centrifugation, washed and resuspended in 1.3% sodium chloride solution and used for agglutination reactions with antiserum of known titer. Table I represents variation in agglutination titer of a homologous antiserum (rabbit) using these organisms as antigens. The titers were markedly reduced in the case of organisms previously suspended in concentrations of 0.85% sodium chloride and less. This is in agreement with Oeding (3) who used both autoclaved and live staphylococci in 0.85% sodium chloride. On the other hand, little reduction of titer occurred when the organisms were previously suspended in solutions of 1.3% sodium chloride and greater. Thus the agglutinating antigen appears to be soluble in salt concentrations of 0.85% and less.

To confirm this, the respective supernatant solutions in which the organisms were originally suspended were cleared by centrifugation and passage through a Seitz filter and were used in the standard antibody absorption test. For final titering, unwashed organisms suspended in 1.3% sodium chloride constituted the antigen. Table II shows the typical results from such a test. Agglutinin is absorbed by the supernatant solutions of suspensions of organisms in concentrations of sodium chloride at 0.85% and below, and remains practically unaffected in concentrations at and above 1.3%.

TABLE I. Effect of Previous Exposure of Staphylococci to Various Concentrations of NaCl upon Their Agglutination by Immune Rabbit Serum.

| Conc. of NaCl to which organisms were exposed (%) | Max agglutination titer of organisms after exposure |
|---|---|
| 0 (distilled water) | 80 |
| .85 | 0 |
| 1.3 | 320 |
| 3.4 | 320 |
| Unexposed | 640 |

TABLE II. Absorption of Agglutinins from Immune Rabbit Serum by Washings of Staphylococci with Various Salt Concentrations.

| Absorbing antigen (washing) according to its conc. of NaCl (%) | Max agglutination titer of antiserum after absorption |
|---|---|
| 0 (distilled water) | 40 |
| .85 | 0 |
| 1.3 | 640 |
| 3.4 | 640 |
| Unabsorbed | 1280 |

It appears, therefore, that the change in agglutination demonstrated is related to the release of antigen into the solution since it is demonstrable there.

*Summary.* Optimal concentration of sodium chloride for quantitative evaluation of staphylococcal agglutinins was 1.3% to 1.7%. This applies to rabbit and human antisera. At this concentration, macroscopic technics are reliable and spontaneous agglutination is inhibited. The quality of the reaction is best at these concentrations for human antisera, while in rabbit antisera, it is best at concentrations of 3.2% sodium chloride. Concentrations below the optimal dissolves the antigen to such extent that macroscopic agglutination is definitely decreased.

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## Effects of Storage on Serum Non-Esterified Fatty Acid Concentrations.* (25371)

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Although Dole(1) pointed out that extracts for non-esterified fatty acids (NEFA) should be prepared "as soon as possible after separation of plasma," no studies on possible changes in NEFA concentrations under various storage conditions have been reported. The present study illustrates that such storage, even in the frozen state, is accompanied by steadily increasing NEFA concentrations.

*Methods.* Fasting blood samples were obtained from 4 randomly selected patients, and from 2 patients with idiopathic hyperlipemia. Hyperlipemic blood samples also were obtained from 2 patients following intravenous infusion of a fat emulsion.† All blood samples were allowed to clot for approximately one hour, and the sera separated. Aliquots were promptly analyzed for NEFA by the method of Dole(1), and for triglyceride by the method of Forbes and Forbes (in preparation for publication). The latter was carried out in order to know the concentration of triglyceride substrate available for possible lipoprotein lipase action. Additional samples of the same sera were promptly separated for storage at −15°C, + 10°C, and room temperature (approximately + 25°C), and were subsequently analyzed for NEFA by the method of Dole (1) on days 1, 3, 8, 15, and 30 (days 1 and 3 only for room temperature stored sera).

*Results.* The initial (zero day) values for serum triglyceride fatty acids and NEFA for each patient are shown in Table I. Fig. 1 presents the effects of storage at the 3 temperatures on NEFA concentrations. Even in the frozen state, NEFA concentrations consistently rose in the first 24 hours, in one case to +63%. Increasing the storage temperature to +10°C and +25°C augmented these rises. In all of the frozen sera and in 5 of 8 sera stored at +10°C, transient decreases in NEFA following the initial rises were observed between days 3 and 15. In sera containing "artificial" chylomicrons from infused fat emulsion, enormous rises during storage oc-

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† Lipomul, supplied by The Upjohn Co.

TABLE I. Patient Groups and Zero Storage Day Values of Serum Triglyceride and Non-esterified Fatty Acids.

| Group | Age | Diagnosis | Serum triglyceride fatty acids (meq/l) | Serum non-esterified fatty acids (meq/l) |
|---|---|---|---|---|
| No hyperlipemia (randomly selected individuals) | 67 | Arteriosclerotic heart disease | 3.98 | .62 |
| | 63 | Hypertensive " " | 5.62 | .72 |
| | 63 | Essential hypertension | 4.41 | .53 |
| | 33 | Rheumatic heart disease | 2.35 | .90 |
| "Physiological" hyperlipemia | 47 | Idiopathic hyperlipemia | 15.43 | 1.12 |
| | 33 | *Idem* | 13.51 | 1.22 |
| "Artificial" hyperlipemia (fat emulsion infusion) | 37 | Arteriosclerotic heart disease | 39.53 | 1.06 |
| | 34 | Simple obesity | 17.56 | .99 |

curred except when the sera were frozen in which case the rises were moderate. Triglyceride from "artificial" chylomicrons therefore appears to be particularly susceptible to lipolysis with resultant release of NEFA.

Since it may be desirable at times to delay analysis, it has been found that refrigerated heptane extracts prepared in the usual manner for NEFA analysis are stable for at least 10 days, within a range of <±3%.

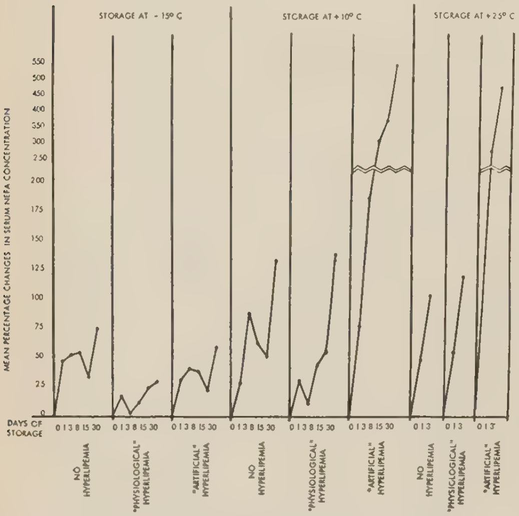

FIG. 1. Effects of storage time and temperature on serum NEFA concentrations, expressed as mean percentage changes from zero day value. Three types of sera at 3 storage temperatures are illustrated. (Note change in vertical scale dimensions for upper portion of Figure.)

*Discussion.* Extracts for serum NEFA analyses should be prepared promptly to reduce *in vitro* lipolysis to a minimum. The data do not distinguish between lipolysis of enzymatic or of physico-chemical origin; it is reasonable to postulate however that such lipolysis is in part due to serum lipoprotein lipase. The explanation for the transient decrease in NEFA between the 3rd and 15th day observed in some of the sera is not evident. Because of the excellent reproducibility of Dole's NEFA method, we do not feel that the transient decreases are due to methodological error.

*Summary.* The effects of storage of serum at various temperatures on NEFA concentrations have been studied. Appreciable increases in NEFA occurred within 24 hours even in frozen sera. Refrigerated heptane extracts of sera for NEFA analysis are stable for at least several days. Sera for NEFA analyses should be extracted promptly after collection.

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# Propagation of Measles Virus in Non-Primate Tissue Culture. I. Propagation in Bovine Kidney Tissue Culture. (25372)

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Propagation of measles virus in human and primate tissue culture(1,2) as well as in chick embryo tissue culture(3) has been described. Attempts by Warren and Cutchins(4) to grow this virus in bovine kidney tissue culture (BKTC) were unsuccessful. This paper describes a method whereby measles virus can be propagated in BKTC, producing a cytopathogenic effect (CPE) typical of that for measles in other tissue cultures(5).

*Material and methods. Virus.* The Edmonston strain(6) of measles virus used was obtained from Enders.* This virus had been propagated 24 times in human kidney and 27 times in human amnion tissue culture (HATC) in Enders' laboratory. After 8 additional passages in HATC in our laboratory, a large seed pool of virus was prepared for the following experiments. *Tissue culture procedure.* BKTC was prepared by suspending 1 part of trypsinized kidney cortex cells from 8-9-months-old bovine fetuses in 200 parts of medium consisting of 0.5% lactalbumin hydrolysate, 5% horse or calf serum, and Earle's basic salt solution containing 200 units of penicillin and 200 μg of streptomycin/ml. This mixture was dispensed in 1 ml amounts in culture tubes and the medium changed at weekly intervals. Human heart cells (Girardi)† (HHTC) were used as indicator of virus in titration and identification tests; 200,000-500,000 cells/ml in Eagle's basal medium (EBM) plus 10% calf serum were dispensed into tubes. Regular medium changes were performed at 3-4 day intervals with EBM plus 2% calf serum. HATC were prepared according to the method of Zitcer *et al.*(7); 300,000-500,000 cells in 1 ml of EBM plus 10% calf serum were dispensed/culture tube, and handled as described for BKTC, using EBM plus 10% calf serum as maintenance medium. *Complement fixation (CF) test.* Tissue culture fluid from measles-infected HATC, inactivated at 56°C for 30 minutes, was used as antigen. Two units each of complement, antigen, hemolysin, plus 2% sheep cells, each in 0.2 ml amounts, were employed in the test. Sera inactivated twice at 56°C for 30 minutes were used in 2-fold dilutions. Complement fixation was carried out at 4°C overnight, after which the hemolytic system was added, with incubation at 37°C for 30 minutes. Endpoints were determined at 50% fixation of complement. Control antigens from non-infected HATC were included in each test. *Neutralization test.* A mixture of equal amounts of serum or serum dilutions and virus in final concentration of approximately 100 tissue culture infectious doses 50% ($TCID_{50}$) was incubated for 1 hour at room temperature, and 0.2 ml inoculated into each of several culture tubes. The final reading was recorded when approximately 100 $TCID_{50}$ were present in the simultaneous virus control titration. *Monkeys.* Cynomolgus monkeys of either sex were used, after an isolation period of 4 weeks following arrival from the Philippines. Their sera were tested for measles CF antibodies immediately and 4 weeks later. Only monkeys free of measles antibodies were used. After inoculation, the monkeys were observed daily for rash or other signs of illness, and rectal temperatures were taken. Serum specimens were drawn at weekly intervals for antibody studies. *Viremia and throat cultures.* Blood specimens for virus isolation were taken every 2nd day for 15 days, beginning with 3rd day post-inoculation. Whole, heparinized blood in amounts of 1.5-2 ml was inoculated into 2-5 tubes, using HATC and/or HHTC. Throat cultures were taken at the same intervals by swabbing the pharynx with sterile cotton which was then submerged and agitated vigorously in 2 ml of tissue culture fluid containing 2,000 units of penicillin, 2,000 μg of streptomycin,

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* The authors express appreciation to Dr. J. F. Enders, Children's Medical Center, Boston, Mass., for supplying this strain of measles virus.

† Obtained from Microbiological Assoc.

TABLE I. Amount of Virus Present in Tissue Culture Fluid in the 1st Passage of Measles Virus in BKTC at Different Times, as Determined by Titration in HHTC.

| Titer in logs/0.2 ml | Post-inoculation day | Titer in logs/0.2 ml | Post-inoculation day |
|---|---|---|---|
| 2.3 | 14 | 2.5 | 51 |
| 1.8 | 23 | 1.8 | 59 |
| 2.3 | 37 | 2.5 | 81* |
| 2.5 | 44 | 3.5 | 81† |

* Tissue culture fluid only.
† After disrupting cells by freezing and thawing.

and 50-100 units of mycostatin/ml. After 30 minutes at room temperature, the cotton was squeezed out and discarded. The fluid was inoculated in 0.2 ml amounts into each of 3-5 tubes. HATC was observed for approximately 27 days, and HHTC for 14 days. One blind passage was performed. Isolations were identified by serum-neutralization test.

*Results.* Each of 6 BKTC tubes was inoculated with 0.5 ml of virus. Three tubes were harvested at 14 days after freezing and thawing, even though no CPE could be noted, and 0.5 ml of fluid and cell mixture transferred into each of several new BKTC. Additional transfers were usually made at 14-16 day intervals, regardless of a CPE, and are referred to as blind passage line (b-line). In the remaining 3 cultures, CPE was observed 70 days after inoculation. Fluid from these cultures was tested for virus in HHTC at different intervals. Production and release of virus were continuous, since amount of virus in the fluid was relatively constant during 81-day period, despite 11 medium changes after inoculation (Table I). On 81st day, although 10%-20% of the cells appeared unaffected, a passage was performed in the manner described for the b-line. This initiated a series of passages in BKTC, referred to as normal, or "n" line, in which transfers were made, when at least 50% of the cells exhibited a CPE. The time required for appearance of CPE was considerably shortened after a few passages (Table II).

TABLE II. Appearance of Definite CPE at Different Passage Levels in BKTC in Relation to the Amount of Virus Inoculated.

| Passage level (n-line) | Day post-inoc. CPE appeared | Amt of virus in 0.5 ml inoc. as titered in HHTC, log |
|---|---|---|
| 1 | 70 | 3.9 |
| 2 | 24 | 3.9 |
| 3 | 8 | 4.7 |
| 4 | 15 | 4.2 |

*Cytopathic effect.* Cytological changes observed in infected cultures resembled those described by Milovanovic *et al.*(5) for measles CPE in HATC, and were characterized by formation of syncytium-like, or multinucleated giant cells. Eosinophilic nuclear inclusions were seen, as well as strongly eosinophilic amorphous material in the cytoplasm. A comparison of normal and infected BKTC cells can be seen in Fig. 1. In the b-line, 8 passages were required before a fairly consistent CPE occurred (Table III). The 16th b-line passage in BKTC yielded $10^{4.3}$ $TCID_{50}/0.2$ ml of virus when titrated in HHTC, which indicated that the virus multi-

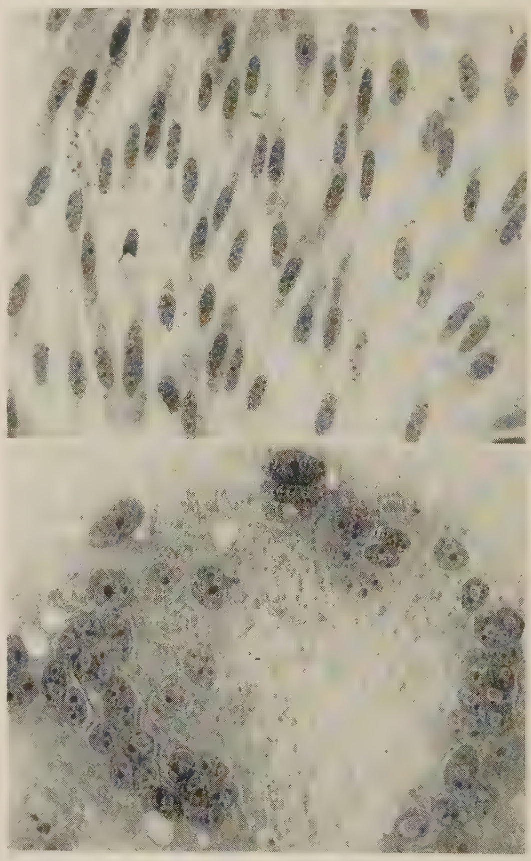

FIG. 1. Comparison of normal bovine kidney tissue culture (above) with a similar culture (below) showing CPE following inoculation with measles virus. Stained with hematoxylin eosin, magnification 192×.

TABLE III. Appearance of a Definite CPE at Different Passage Levels of the Blind-Passage Line in BKTC.

| Passage level (b-line) | Day post-inoc. CPE appeared* | Amt of virus in 0.5 ml inoc. as titered in HHTC, log |
|---|---|---|
| 1-4 | 0 | +† |
| 5 | 14 | 3.4 |
| 6 | 0 | 3.4 |
| 7 | 0 | + |
| 8 | 0 | + |
| 9 | 14 | 3.9 |
| 10 | 11 | 4.2 |
| 11 | 10 | 3.2 |
| 12 | 0 | 2.7 |
| 13 | 13 | 3.7 |
| 14 | 13 | 3.9 |
| 15 | 0 | 3.9 |
| 16 | 7 | 4.2 |
| 17 | 7 | 4.7 |
| 18 | 5 | 5.7 |

* Tubes passed after approx. 14-16 days.

† Virus present as tested in HHTC, but not titrated.

plied in the BKTC, since this endpoint of titration represents a dilution of $10^{-15.5}$ of original inoculum which titrated only $10^{-3.5}$/0.2 ml.

TABLE IV. Results of Neutralization Test with Human and Monkey Measles Sera and 100 $TCID_{50}$ after 4th Normal Bovine Measles Virus in HHTC.

| Serum 2-fold dilution | Acute or pre-inoc. | Convalescent, or post-inoc. |
|---|---|---|
| Human | 1:13 | 1:604 |
| Monkey | 0 | 1:256 |

*Identification.* Virus propagated in BKTC, when tested at different passage levels in HHTC or HATC, still demonstrated the typical characteristics of measles virus. The 4th BKTC n-line virus was identified by neutralization test in HHTC, using acute and convalescent sera from a measles patient, and also pre- and post-sera from a monkey inoculated with Edmonston strain of measles virus passed in HATC only. The virus was neutralized to any significant degree only by convalescent and post-inoculation sera (Table IV). The 15th b-line virus was also identified by a neutralization test in BKTC, HATC and HHTC, using monkey pre- and post-inoculation sera. Post-inoculation sera neutralized the virus in all 3 tissue culture systems, while the negative sera had no inhibitory effect.

*Tests in monkeys.* Two monkeys were inoculated with 2nd passage n-line virus, and 3 with 20th passage b-line virus. Each monkey received 1 ml of undiluted, infected tissue culture fluid intramuscularly. No evidence of disease occurred, but the results (Table V) showed that virus could be isolated from throat and blood on several occasions.

*Discussion.* The above experiments show conclusively that measles virus can be propagated in BKTC, with production of a typical CPE which can be prevented by known measles immune sera. This study indicates, however, that to obtain a CPE for some viruses in certain tissue cultures either a prolonged period of observation, or more than 1 or 2 blind subcultures are necessary. Furthermore, it emphasizes the value of a good virus detection system, such as HHTC, for this particular strain of virus.

Inoculation of monkeys with BKTC-propagated virus provided further evidence that the CPE agent was measles, by development of measles complement-fixing and neutralizing antibodies, even though no illness or rash was observed. The fact that the virus could be recovered from the throat and blood of inoculated monkeys might be interpreted as a sign of virulence, but the final answer to this question can only by found by inoculation of

TABLE V. Intramuscular Inoculation of Bovine Kidney-Propagated Measles Virus into Cynomolgus Monkeys.

| Passage No. and amt of virus inoc.* | Virus recovery, days post-inoc. | | | | Antibody | | | |
|---|---|---|---|---|---|---|---|---|
| | Human amnion T.C. | | Human heart T.C. | | C.F. | | Neutralization | |
| | Blood | Throat | Blood | Throat | Pre | Post | Pre | Post |
| 2nd 5.0 | 5 | N.T. | N.T. | N.T. | 0 | 1:256 | 0 | 1:320 |
| ” ” | 3, 7, 9 | ” | ” | ” | 0 | >1:1024 | 0 | 1:320 |
| 20th 5.2 | 3, 5, 7 | 0 | 3, 5, 7, 9 | 7, 9 | 0 | 1:512 | 0 | 1:32 |
| ” ” | 3, 5, 7 | 7 | 3, 5, 7 | 5, 9 | 0 | 1:2042 | 0 | 1:320 |
| ” ” | 5, 7 | 5, 7 | 5, 7 | 5, 7 | 0 | 1:1024 | 0 | 1:64 |

* In logs. N.T. = not tested.

susceptible humans. Regardless of the possible virulence of the present BKTC virus, propagation of measles virus in this tissue culture system may prove valuable as a means of further modification. It would also permit economical production of large quantities of measles virus for use in either modified or killed vaccines. Bovine embryonic tissue would be superior to monkey tissue since it is more readily available and, to our knowledge, contains no latent viruses; it further would be superior to chick embryo tissues to which many people are already sensitive.

*Summary.* 1. The Edmonston strain of measles virus was propagated in BKTC for 20 passages, and produced a CPE typical of measles, characterized by multinucleated giant cells and eosinophilic intranuclear inclusion. 2. Identification of this cytopathogenic agent was confirmed by neutralization test with known measles immune serum. 3. BKTC-propagated virus was inoculated intramuscularly into susceptible cynomolgus monkeys without causing clinical illness, but the virus could be recovered from throat and blood. All inoculated monkeys developed complement-fixing and neutralizing measles antibody. The significance of these results is discussed.

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## Failure to Transfer Delayed Tuberculin Sensitivity from Human to Animal Species.* (25373)

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Transfer of delayed cutaneous sensitivity to tuberculin and to other materials with leucocytes obtained from specifically sensitive donors has been reported by Chase in the guinea pig and by Lawrence in man(1). In animals, the transfer of sensitivity is successful only when intact leucocytes are employed. However, in man, it has been shown that the factor in sensitive leucocytes concerned with transfer of delayed sensitivity retains its activity following leucocyte disruption and enzymatic treatment(2). This finding has been confirmed recently by Freedman, Fisher and Cooke(3). A valuable approach to the study of transfer factor would be provided by transfer of delayed sensitivity from man to animals —particularly if leucocyte extracts could be used. Lawrence, as well as Rammelkamp, and Thomas and Stetson(4) have repeatedly attempted to transfer tuberculin sensitivity to guinea pigs and rabbits with intact as well as disrupted human leucocytes obtained from sensitive donors. Their results have been consistently negative. The present study is based on the assumption that species barrier in transfer of sensitivity might be crossed more readily within a closer evolutionary relationship, as exists between man and the monkey. This report describes a series of attempts to

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[*] The opinions or assertions contained herein are the private ones of the authors, and are not to be construed as official, or reflecting the views of the Navy Department or of the Naval Service at large.

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transfer delayed tuberculin sensitivity from man to monkey, and from man to rabbit by means of peripheral blood leucocytes obtained from strongly tuberculin-sensitive human donors.

*Materials and methods.* 1. *Selection of leucocyte donors and recipients.* The staff of the Naval Biological Laboratory were screened with a tuberculin patch (Vollmer). The most sensitive reactors, each giving a raised, vesicular and intensely erythematous reaction at test site 48 hours after application of the patch, were selected as donors. These were subjects *H, T, C,* and *D.* The recipients included 10 monkeys (*Macacus mulatta*), weighing 2-7 kg each, and 8 white male albino rabbits weighing 0.8-1.0 kg each. All animals gave a negative reaction to 0.1 ml of diluent solution containing 0.005 mg PPD (Purified Protein Derivative)(4) intradermally. 2. *Isolation of leucocytes.* Each blood donation (400 ml) is collected in a plastic Fenwal bag to which are added 100 ml of aqueous solution containing 0.05 g/ml of bovine fibrinogen (Fraction I, Armour). The bag contents are mixed, and the bag suspended in incubator at 37°C for 45 minutes. Sedimented erythrocytes are discarded, and the supernatant plasma suspension of leucocytes is collected in graduated conical centrifuge tubes, and spun at 2000 g 15 minutes. Volume of packed leucocytes in each tube is recorded, the supernatant plasma is discarded, and the leucocytes pooled and resuspended in sterile pyrogen-free normal saline. 3. *Methods of transfer.* Three routes of administration were employed to transfer tuberculin sensitivity with leucocytes collected from 4 human donors. Several methods of leucocyte preparation were used: a) *Intradermal and subcutaneous route. First method of leucocyte preparation*: 0.84 ml of leucocytes from donor *H* were suspended in 4 ml of normal saline, and injected in 0.3 ml aliquots into the left thigh of recipient monkey (R1). A second monkey recipient (R2) received a similar volume of *H* plasma. *Second method*: 0.375 ml of leucocytes from donor *D,* suspended in 2 ml of normal saline solution, were incubated at 37°C for 30 minutes with 1 ml of normal saline solution containing 50 γ of PPD. 0.30 ml of resulting mixture (containing approximately 0.04 ml leucocytes) were injected into the next 3 monkey recipients (R3, R4, R5). *Third method*: 0.375 ml of leucocytes obtained from donor *D,* suspended in 2 ml of normal saline solution, were subjected to 7 cycles of rapid alternate freezing (95% alcohol-dry ice mixture) and thawing (37°C water bath). One ml of normal saline solution containing 50 γ of PPD was added to the disrupted leucocyte suspension, and the resulting mixture was incubated at 37°C for 30 minutes. Following incubation, 0.30 ml of this material was injected intradermally into 2 additional monkeys (R6 and R7). b) *Intraperitoneal route.* 0.72 ml of leucocytes obtained from donor *T* were suspended in 13 ml of normal saline solution, and injected intraperitoneally into monkey recipient (R8); another monkey (R9) received a similar volume of *T* plasma intraperitoneally. c) *Intravenous route.* 1.07 ml of leucocytes obtained from donor *C* were suspended in 12 ml of normal saline solution, and administered intravenously to final monkey (R10). No untoward reaction was noted to any of the methods of administration of human leucocytes employed, with or without added PPD. 4. *Concurrent study with rabbits.* Two groups of 4 rabbits each were included in this study. The first group (Rabbits 1-4) each received intradermal injections of 0.30 ml of *D* whole leucocyte-PPD mixture described in section 3A. The second group (Rabbits 5-8) each received intradermal injections of 0.30 ml of disrupted *D* leucocytes-PPD mixture described in section 3A.

*Results.* Monkeys receiving whole leucocytes, either intradermally, intraperitoneally, or intravenously (R1, R8 and R10), as well as control running mates (R2 and R9) were tested intradermally with 0.005 mg PPD in 0.1 ml diluent solution, at 24 hours, and thereafter at weekly intervals for 5 weeks following cell transfer. Neither test nor control animals developed tuberculin sensitivity during this period.

Monkeys which had receivd intradermal injections of mixtures of intact leucocytes and PPD (R3, R4 and R5), as well as the group which had received intradermal injections of mixtures of leucocyte extract and PPD (R6

TABLE I. Attempted Transfer of Tuberculin Sensitivity to Monkeys with Human Leucocytes.

| | | | Skin reactivity to tuberculin after transfer, at: | | | | | |
|---|---|---|---|---|---|---|---|---|
| Subject | Material used for transfer | Route of administration | 24 hr | 1 wk | 2 wk | 3 wk | 4 wk | 5 wk |
| R 1 | .84 ml intact leucocytes | Intrad. & subcut. | 0 | 0 | 0 | 0 | 0 | 0 |
| R 2 | Plasma from same donor | *Idem* | 0 | 0 | 0 | 0 | 0 | 0 |
| R 3 | .04 ml intact leucocytes incubated with tuberculin | " | — | 0 | 0 | — | — | — |
| R 4 | *Idem* | " | — | 0 | 0 | — | — | — |
| R 5 | " | " | — | 0 | 0 | — | — | — |
| R 6 | .04 ml disrupted leucocytes incubated with tuberculin | " | — | 0 | 0 | — | — | — |
| R 7 | *Idem* | " | — | 0 | 0 | — | — | — |
| R 8 | .72 ml intact leucocytes | Intraper. | 0 | 0 | 0 | 0 | 0 | 0 |
| R 9 | Plasma from same donor | " | 0 | 0 | 0 | 0 | 0 | 0 |
| R10 | 1.07 ml intact leucocytes | Intrav. | 0 | 0 | 0 | 0 | 0 | 0 |

and R7), were similarly skin tested one and 2 weeks after transfer. None of the animals developed tuberculin sensitivity. The results of this experiment are summarized in Table I.

Rabbits which had received intradermal injections of either mixtures of intact leucocytes and PPD (Rabbits 1-4) or mixtures of leucocyte extract and PPD (Rabbits 5-8) were also skin tested with the same concentration of PPD, one and 2 weeks after cell transfer. No animals in this group developed reactivity to tuberculin during this period. The results of this experiment are summarized in Table II.

*Discussion.* Our results suggest that, within the limitations of the experimental approach employed, it is not possible to transfer tuberculin sensitivity from man to monkey or from man to rabbit, by means of leucocytes obtained from peripheral blood of specifically sensitive donors. Despite variations in route of administration and in method of treatment of the leucocytes, all recipients remained tuberculin negative upon repeated subsequent skin testing.

The significance of these results may be weakened by the absence of specific information concerning whether delayed sensitivity can be transferred from monkey to monkey by this method. However, as monkeys are known to have the capacity to develop cutaneous tuberculin sensitivity(5), it is not likely that this species would differ markedly from guinea pig, rabbit, or man, in their response to cellular transfer of sensitivity performed within the species.

*Summary.* This report describes attempts to transfer delayed sensitivity to tuberculin to monkeys and to rabbits by means of peripheral blood leucocytes obtained from sensitive human donors. No such transfers were obtained.

TABLE II. Attempted Transfer of Tuberculin Sensitivity to Rabbits with Human Leucocytes.

| | | | Skin reactivity to tuberculin after transfer at: | |
|---|---|---|---|---|
| Subject | Material used for transfer | Route of admin. | 1 wk | 2 wk |
| 1 | .04 ml intact leucocytes incubated with tuberculin | Intrad. & subcut. | 0 | 0 |
| 2 | *Idem* | *Idem* | 0 | 0 |
| 3 | " | " | 0 | 0 |
| 4 | " | " | 0 | 0 |
| 5 | .04 ml disrupted leucocytes incubated with tuberculin | " | 0 | 0 |
| 6 | *Idem* | " | 0 | 0 |
| 7 | " | " | 0 | 0 |
| 8 | " | " | 0 | 0 |

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# Mechanism of Clot Lysis by Streptokinase and Effects of Epsilon-Aminocaproic Acid.* (25374)

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In past years considerable experimental work, initiated in laboratories of W. S. Tillet (1), was undertaken to investigate the possibility of dissolving intravascular thrombi in humans by intravenous administration of streptokinase (SK). These and other investigators employed plasmin, prepared by activation of human plasminogen with SK, as the lytic agent, and recently reviewed by Sherry *et al.*(2). It is recognized that either form of treatment may cause certain undesirable side-effects(2) from induced hyperplasminemia, among which may be listed (a) destruction of fibrinogen and plasminogen, (b) destruction of some components involved in blood coagulation, (c) destruction of other physiologically active polypeptides and (d) elevation of antithrombin levels. Obviously it would be desirable to prevent or minimize the untoward systemic effects induced by hyperplasminemia, without interfering with dissolution of the thrombus. Epsilon-aminocaproic acid ($\epsilon$-ACA), first reported to be a plasmin inhibitor (3), was recently shown by Ablondi and Hagan(4) to be a far more potent inhibitor of plasminogen activation. Subsequent studies confirmed and extended this observation to show that $\epsilon$-ACA inhibited both SK and urokinase activation of plasminogen(5,6). Our more recent studies demonstrated that $\epsilon$-ACA does not adsorb to a fibrin clot, a situation analogous to that found by Johnson, *vide infra,* for humoral inhibitors of fibrinolysis. It seemed worthwhile, therefore, to determine (a) the effect of $\epsilon$-ACA on dissolution of a clot suspended in plasma and (b) its simultaneous effect on plasminogen activation in plasma. Our purpose here is to present data which demonstrate that, under certain conditions *in vitro,* $\epsilon$-ACA can effectively inhibit activation of plasminogen by SK in plasma without markedly inhibiting the lysis of a clot immersed in such plasma.

*Methods.* $I^{131}$-labeled clots fortified with plasminogen were prepared as follows: Twelve cc of 0.9% saline were added to 1 cc aliquots of human plasma followed by 0.1 cc of a solution of $I^{131}$-labeled fibrinogen prepared by the method of Mihalyi and Laki(7). One-tenth cc human plasminogen containing 4000 units of proactivator(8), and prepared by the method of Kline(9), was then added. Clotting was effected at pH 7.4 by addition of 1.25 units of thrombin (Parke-Davis) in 0.1 cc volume. As clots formed they were collected by carefully winding onto glass capillary tubes (melting point tubes) to which they became tightly fixed. As much fluid as possible was squeezed from the adhered clot by pressing against inner wall of test-tube. The radioactive content of each clot was determined in a well type scintillation counter

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TABLE I. Inhibition of SK Activation of Plasminogen by ε-ACA in Plasma and Lack of Inhibition of Clot Lysis (Plasminogen Fortified Clot).

| | | Clot dissolution | | | | Fibrin plate assay zones of lysis† ($d^2$ in mm) | |
|---|---|---|---|---|---|---|---|
| SK (u/ml) | ε-ACA conc. (M) | Clot radioactivity (cpm) at 0 time | Clot radioactivity (cpm) at 1 hr | Plasma radioactivity (cpm) at 1 hr | % of initial clot counts in plasma at 1 hr* | Unheated | Heated |
| 0 | 0 | 15920 | 14220 | 1480 | 9.3 | 0 | 0 |
| 50 | 0 | 13770 | 10040 | 3820 | 28.2 | 64 | 9 |
| 100 | 0 | 14670 | 10040 | 4920 | 33.7 | 169 | 16 |
| 150 | 0 | 13220 | 8070 | 5290 | 39.5 | 225 | 25 |
| 200 | 0 | 12700 | 5780 | 6570 | 51.5 | 289 | 64 |
| 0 | .025 | 17720 | 15660 | 1200 | 5.6 | 0 | 0 |
| 50 | ” | 11730 | 9060 | 2535 | 21.6 | 0 | 0 |
| 100 | ” | 13800 | 9520 | 5020 | 35.9 | 0 | 0 |
| 150 | ” | 14000 | 8620 | 5520 | 39.2 | 0 | 0 |
| 200 | ” | 15300 | 5940 | 10370 | 68.0 | 0 | 0 |

* Sum of counts on clot at 1 hr and in plasma at 1 hr equals 95-101% of initial clot count.

† .03 ml of each plasma reaction mixture was assayed by the procedure described by von Kaulla(10).

after which the clots were immersed in 10 cc 0.03 M phosphate buffer, pH 7.4 at 5°C until used. No radioactivity was washed from the clot during this period. Plasma incubation medium containing SK or SK plus ε-ACA was prepared as follows: Sodium chloride was added to an aliquot of human plasma to give a concentration of 0.025 M. ε-ACA was added to a second aliquot to give the same molar concentration. To 2 cc aliquots of plasma, containing either ε-ACA or NaCl, varying amounts of SK in 0.1 cc of buffer were added to give 0, 50, 100, 150 and 200 units of SK (Varidase®)/cc. All reactants were maintained at 3-5°C to minimize instability of activator. Immediately before immersion of a radioactive clot 0.03 cc sample of each plasma reaction mixture was withdrawn and assayed for activator and plasmin activities using fibrin plate method of Astrup and Mullertz as modified by von Kaulla(10). Zones of lysis on the unheated plate served as a measure of activator and/or plasmin activity while zones of lysis on the heated plate were a measure of plasmin activity only. A prepared radioactive clot was then added to each plasma reaction mixture in a 75 × 10 mm Wassermann tube. The reaction mixtures were then warmed to 37°C in a constant-temperature water bath and incubated one hour. Then the clots were removed, squeezed free of plasma by pressing against the inner side of the tubes, and radioactivity determined in both the clots and plasma.

*Results* are shown in Table I. Little inhibition of clot lysis occurred in the presence of ε-ACA although activation of plasminogen was completely inhibited in the plasma incubation mixtures as measured by the fibrin plate method. Essentially similar results were obtained when the concentration of ε-ACA was increased to 0.05 M (data not shown).

It was of further interest to repeat the experiment with clots which were not fortified with plasminogen. Radioactive clots were prepared as described above, except that no exogenous plasminogen was added before clotting. Since rate of clot lysis was much slower with clots prepared in this manner, incubation time was extended to 2½ hours. The results are shown in Table II. They again demonstrate a complete inhibition by ε-ACA of plasminogen activation in plasma. However, partial inhibition of clot lysis is also apparent.

*Discussion.* These experiments demonstrate that clot lysis can occur in plasma in which no detectable plasmin is present because of inhibition of its formation by an inhibitor of plasminogen activation, ε-ACA. In agreement with the findings of Alkjaersig *et al.*(11) the data also demonstrate that plasminogen is adsorbed (or entrapped within) a clot as it forms and that the rate of clot lysis induced by SK is a function of clot plasminogen concentration.

Much attention has been devoted to the apparently greater fibrinolytic rather than fibrinogenolytic activity of plasmin. Studies

TABLE II. Inhibition of SK Activation of Plasminogen in Plasma by ε-ACA with Partial Inhibition of Clot Lysis (Unfortified Clot).

| | | Clot dissolution | | | | | |
|---|---|---|---|---|---|---|---|
| SK (u/ml) | ε-ACA conc. (M) | Clot radioactivity (cpm) at 0 time | Clot radioactivity (cpm) at 2½ hr | Plasma radioactivity (cpm) at 2½ hr | % of initial clot counts in plasma at 2½ hr* | Fibrin plate assay zones of lysis† ($d^2$ in mm) Unheated | Heated |
| 0 | 0 | 12870 | 12550 | 215 | 1.5 | 0 | 0 |
| 50 | 0 | 11300 | 9270 | 2000 | 17.7 | 64 | 16 |
| 100 | 0 | 12230 | 9090 | 2860 | 22.8 | 196 | 16 |
| 150 | 0 | 11600 | 8810 | 2510 | 21.8 | 256 | 36 |
| 200 | 0 | 11450 | 8270 | 3090 | 26.4 | 361 | 81 |
| 0 | .025 | 11900 | 11050 | 190 | 1.7 | 0 | 0 |
| 50 | " | 11750 | 11450 | 610 | 5.2 | 0 | 0 |
| 100 | " | 12210 | 11300 | 910 | 7.2 | 0 | 0 |
| 150 | " | 11750 | 11450 | 1060 | 9.1 | 0 | 0 |
| 200 | " | 12700 | 11050 | 1130 | 9.2 | 0 | 0 |

* Sum of counts on clot at 2½ hr and in plasma at 2½ hr equals 95-101% of initial clot count.

† .03 ml of each plasma reaction mixture was assayed by the procedure described by von Kaulla(10).

suggest that this phenomenon is due to adsorption of plasmin on fibrin as it forms, thereby resulting in a concentraion of enzyme on the substrate to be digested(12). Results of our experiments are in agreement with this interpretation (Unpublished). Of further interest, in this connection, is the observation of Johnson that inhibitors of fibrinolysis are not adsorbed to fibrin (personal communication). Indeed, Astrup(13) suggested that the presence of inhibitors of plasmin in blood interferes with splitting of soluble proteins, including fibrinogen. However, an effect of plasmin on fibrin is still exerted by virtue of its adsorption to fibrin. He states "we can but admire the ingenious way Nature has solved the extremely difficult problem of providing blood with a proteolytic system which exerts its effect only when and where it is needed."

A final speculation on the clinical application of these findings appears pertinent. It is possible that advantage would be gained by the simultaneous administration of an inhibitor of plasminogen activation along with an activator of plasminogen for treatment of thromboembolic disease. It might then be possible to inhibit or minimize the systemic formation of plasmin and thus avoid harmful effects of hyperplasminemia without significantly inhibiting clot dissolution. Experiments to test this hypothesis are underway in experimental animals.

*Summary.* The effects of ε-ACA, a potent inhibitor of plasminogen activation, on *in vitro* pre-formed clot lysis and plasma plasminogen activation by SK have been studied. Complete inhibition of plasma plasminogen activation by ε-ACA has been demonstrated to occur with little concomitant effect on the dissolution of a clot immersed in plasma. Rate of clot dissolution by SK is shown to be a function of clot plasminogen concentration. Potential clinical applications are discussed.

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# Phosphoglucomutase Activity in Hereditary Muscular Dystrophy in Mice.* (25375)

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Activity of phosphoglucomutase, determined in biopsy samples of skeletal muscles of man with progressive muscular dystrophy, is markedly lower than normal when measured without addition of the coenzyme (glucose 1, 6 diphosphate) to the system(1). Bodansky (2) refined the assay method of Najjar(3), adding coenzyme in varying amounts to eliminate distortions in measured phosphoglucomutase activity due to residual coenzyme in the tissue. The present study concerns determination of mutase activity, both with and without added coenzyme, in leg muscles of mice with hereditary muscular dystrophy, whose myopathy is suggestive of progressive muscular dystrophy in man(4).

*Methods and materials.* Pairs of dystrophic and control mice of same age and sex were obtained from Roscoe B. Jackson Memorial Laboratory. Three pairs were 51-56 days old while 6 other pairs were 86-106 days old when sacrificed. Leg muscles were excised from nembutal-anesthetized animals, trimmed of extraneous tissue, frozen with dry ice, and stored at –20° until ready for use. Phosphoglucomutase activity was determined by a modification of the method of Najjar(3), as refined by Bodansky(2). The final reaction volume of 1 ml. included 0.3 ml of 1% aqueous homogenate of muscle and freshly prepared cysteine. Glucose-1-phosphate was obtained from Schwarz Labs., lot #2102. The ice cold homogenate and the substrate-buffer mixture were warmed separately at 37° for 3 minutes prior to combining. After 4 minutes, the reaction was stopped by adding 1 ml of 5N $H_2SO_4$. The mixture was diluted to 5 ml, heated for 7 minutes at 100°, filtered, and 1 ml aliquot taken for P determination(5). The results are reported as mg glucose-6-phosphate P formed/100 mg fresh tissue/hour. Since purified coenzyme was not available, maximum mutase activity was determined(2) by adding to the reaction mixture various amounts of boiled normal muscle homogenate (equivalent to 4, 8, and 12 mg of tissue) prepared according to Cardini *et al.*(6). The relative activities in presence of different amounts of boiled extract containing coenzyme thus permitted extrapolation to activity at infinite coenzyme concentration. The linearity of 1/concentration of coenzyme *vs.* 1/velocity of the reaction, proposed by Sutherland and coworkers(7) and tested by Bodansky(2) for purified coenzyme, was confirmed for addition of boiled homogenate to both crude and crystalline phosphoglucomutase preparations. The non-collagenous protein N (NCPN) content of the homogenates was determined by the method of Lilienthal, *et al.* (8).

*Results.* Table I indicates that phosphoglucomutase activity is significantly decreased in the dystrophic leg muscles, regardless of the basis upon which the activity is measured. The importance of adding coenzyme to the reaction mixture is emphasized by the difference in values for the 13-week-old mice with and without added coenzyme (39% and 68% decreases, respectively). The further but less striking importance of basing results on NCPN rather than on wet weight(8,9) is seen in the difference between the ratios of activity at infinite added coenzyme concentration in 13-week-old mice: 52.0% on wet weight, 60.7% on NCPN. The NCPN content of dystrophic muscles in older mice was 26.2 ± 1.1 mg/g wet weight as opposed to 28.5 ± 1.3 mg/g wet weight in control mice, representing a decrease of 8% in NCPN content of diseased muscles.

*Discussion.* Values in Table I for phosphoglucomutase activity, based on wet weight of tissue and without added coenzyme, are similar to those obtained in previous experiments on 6 pairs of mice 14-18 weeks of age.

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† Medical Student Fellow of Nat. Fn.

TABLE I. Phosphoglucomutase Activity in Dystrophic Mouse Muscle.

| Exp. (No. of mice) | Without added coenzyme |  |  | At infinite coenzyme conc. |  |  |
|---|---|---|---|---|---|---|
|  | Fresh tissue* | NCPN† | Ratio‡ ×100 | Fresh tissue* | NCPN† | Ratio‡ ×100 |
| 8 wk old |  |  |  |  |  |  |
| Dystrophic (3) | 7.4 ± .3 | 2.9 ± .3 | 53.4 ± 5.8 | 23.5 ± 1.8 | 8.8 ± .1 | 74.5 ± 4.8 |
| Control (3) | 14.4 ± 1.9 | 5.5 ± .2 |  | 30.5 ± 2.1 | 11.9 ± .6 |  |
| 13 wk old |  |  |  |  |  |  |
| Dystrophic (6) | 5.7 ± 1.7 | 2.3 ± .7 | 32.0 ± 9.5 | 17.9 ± 2.1 | 7.4 ± .9 | 60.7 ± 4.4 |
| Control (6) | 18.8 ± 1.3 | 6.6 ± .4 |  | 34.2 ± 2.3 | 12.2 ±1.6 |  |
| P (significance) | <.001 | <.001 |  | <.001 | <.01 |  |

* Activity = mg P/100 mg tissue/hr ± stand. error.

† Activity = mg P/mg non-collagenous protein nitrogen/hr ± stand. error.

‡ Ratios of dystrophic/control, based on NCPN results.

This group decreased 65% in mutase activity in leg muscles, and 70% in abdominal muscles, while heart muscle exhibited no significant change. The importance of basing muscle analyses on NCPN has been previously demonstrated(8,9). In the present study, however, there was little difference between enzyme activity ratios of dystrophic to normal muscle based on NCPN and those ratios calculated on wet weight basis. On the other hand, it is very important to add sufficient coenzyme to permit calculation of maximum activity, independent of the coenzyme content of the tissue.

When so calculated, not only is mutase activity increased in both dystrophic and normal muscle as compared to activities recorded in the absence of added coenzyme, but also the activity ratio of dystrophic to normal is raised. This fact suggests that the coenzyme content of the dystrophic muscle is also decreased, only enough coenzyme remaining to permit a much reduced conversion of glucose-1-phosphate. Furthermore, it might be inferred that this loss of coenzyme is relatively greater than the loss of the enzyme: if the opposite were true, the ratio of dystrophic/normal activities would decrease upon addition of coenzyme, and if both enzyme and coenzyme were decreased to the same degree (thus remaining in constant proportions), the ratio in the absence of added coenzyme would be identical to that at infinite coenzyme concentration. Direct determination of amounts of coenzyme in small muscle samples would substantiate this inference(6,10). The role of glucose-1-phosphate kinase in producing the coenzyme(10) should also be investigated.

Although observations on 8-week-old mice are few, the results suggest a progressive decline in mutase activity as the dystrophic mice mature and the myopathy becomes more severe. This corroborates previous reports that changes in some enzyme activity in dystrophic mouse muscle are correlated with age of mice(11).

The decreases in mutase activity in human muscular dystrophy, determined in the absence of added coenzyme and based on wet weight and NCPN, are of the same order of magnitude as those found for dystrophic mice (1). No results calculated at infinite added coenzyme concentration are available for comparison. A slightly smaller decrease in the glycogenolytic enzyme, phosphorylase, has previously been reported for dystrophic mice (12). The decrease in human dystrophy is somewhat more marked(1).

Information of the biochemical constituents of dystrophic and normal mouse muscle is being accumulated with the object of comparing the findings with those observed in clinical dystrophies. Some resemblances and some differences between the two have been found; as yet, however, no definite pattern has emerged to permit broad generalizations(11, 13,14).

*Summary.* A modified method of assaying maximum phosphoglucomutase activity in crude muscle homogenates is presented. Mutase activity is significantly decreased in leg muscles of mice with muscular dystrophy whether determined as total enzyme activity (at infinite coenzyme concentration) or as

residual enzyme-coenzyme activity (in the absence of added coenzyme). Evidence is presented for a decrease in coenzyme content of dystrophic muscle and for a correlation of these enzyme changes with age of mice and severity of myopathy.

The authors thank Dr. Oscar Bodansky for his counsel and generous contribution of crystalline phosphoglucomutase.

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# Effect of Lactose on the Utilization of Phosphorus from Colloidal Phosphate by Chicks.* (25376)

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The effect of lactose on absorption of calcium and phosphorus has been of considerable interest, and a great deal of work has been done on the over-all effects of lactose feeding. Lactose, when included at a level of 25% in the basal diet, increased the amount of calcium and phosphorus absorbed due to formation of lactic acid by fermentation in the intestine(1). Simultaneous feeding of lactose and bone meal to dairy calves resulted in increased calcium retention from bone meal (2). These authors found that feeding a diet containing 25% lactose caused marked lowering of pH of contents of the digestive tract below the duodenum. Inclusion of lactose at a level of 40% in the diets of young chicks of a rachitogenic ration produced a favorable effect on calcium absorption, as well as having a positive influence on maintaining a more acid condition in the entire intestinal tract (3). Colloidal phosphate (soft phosphate with colloidal clay), obtained as by-product in the mining of rock phosphate, has been offered as a phosphorus supplement, to be used in animal rations. Recent workers(4,5, 6) reported that this supplement, when used as sole source of phosphorus in the diet, gave lower growth rate and (percent) bone ash in chicks than was produced by tricalcium phosphate. Since colloidal phosphate is a cheap source of phosphorus, it was our object to study the effect of various levels of lactose on availability of phosphorus from colloidal phosphate.

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† Present address: The Pillsbury Co., Clinton, Iowa.

*Methods.* Three hundred and sixty day-old Vantress x Nichols 108 chicks were randomly divided into 18 groups of 20 chicks (equal number of males and females in each group) and 2 groups were fed on each diet. Composition of basal diet was as follows: ground corn 59.5%, soybean oil meal 23%, dehydrated alfalfa 5.0%, dried whey 3.0%, sodium chloride 0.5%, $MnSO_4$ (70%) 120 mg/lb and vitamin mixture 1.0%. The following vitamins were supplied/lb of diet: vit. A 4540 I.U., vit. $D_3$ 600 I.C.U., riboflavin 2 mg, calcium pantothenate 5 mg, niacin 12.5 mg, vit. $B_{12}$ 4.0 $\mu$g, choline chloride 400 mg and aureomycin hydrochloride 10 mg. The vitamins were premixed with cerelose to give a level of 1 lb/100 lb of diet. The basal diet fed to Group 1 contained 0.34% phosphorus from dibasic sodium phosphate and those of the other 4 groups were supplemented with 0.2% phosphorus from colloidal phosphate. Lactose was added at levels of 0, 5, 10 and 15% of basal diet. Each diet was maintained at the same calorie-protein ratio by adjusting levels of soybean oil meal and ground corn. The calcium:phosphorus ratio was maintained at 2:1 in all diets. The birds were housed in electrically-heated batteries with raised wire floors, and feed and water were supplied *ad lib.* All groups were observed daily and group weights were recorded at weekly intervals. Birds were weighed individually at end of the 4-week experimental period and feed consumption was recorded. Five birds were selected at random from each replication and the left tibia was removed for bone ash determination according to the A.O.A.C. method (7). Intestinal pH was measured with Beckman pH meter, on the same birds used for bone ash determination. Birds were anesthetized and killed with chloroform vapor. The intestine was removed and pH measured at duodenal loop, 4 inches posterior to duodenum, caecum, and 4 inches anterior to caecum, by procedure similar to that decided by March *et al.*(8). In a second experiment, lactose was added as supplement to a purified diet which consisted of the following ingredients: Purified soybean protein 25%, soybean oil 2.5%, vit. mixture 1.41%, and mineral mixture 2.37%. Cerelose was added to make the diet 100%, after addition of calcium carbonate and phosphorus supplements. The Ca:P ratio was maintained at 2:1. The following vitamins were added/kg of diet: thiamin HCl 10 mg, riboflavin 10 mg, *D*-calcium pantothenate 20 mg, pyridoxine HCl 6 mg, inositol 500 mg, biotin 0.2 mg, niacin 75 mg, folic acid 4 mg, vit. A 10,000 I.U., vit. $D_3$ 2000 I.C.U., $\alpha$-tocopheryl acetate 20 I.U., choline chloride 1.5 g, menadione sodium bisulfite 0.5 g, vit. $B_{12}$ 20 $\mu$g, methionine 7500 mg, glycine 3500 mg, aureomycin HCl 10 mg and BHT (25%) 500 mg. The mineral supplement consisted of the following ingredients/kg of diet: $MnSO_4 \cdot H_2O$ 1.1 g, $MgSO_4 \cdot 7H_2O$ 5.7 g, KI 0.04 g, NaCl 9.5 g, $FeC_6H_5O_7 \cdot 5H_2O$ 1.6 g, $CuSO_4 \cdot 5H_2O$ 0.016 g, $ZnCl_2$ 0.014 g, $CoCl_2$ 0.005 g and KCl 5.72 g. Four groups of chicks were fed 0.4% phosphorus as dibasic sodium phosphate, and 4 groups were fed 0.4% phosphorus as a combination of dibasic sodium phosphate and colloidal phosphate, each supplying 0.2% phosphorus to the diet. The diets were supplemented with lactose at levels of 0, 5, 10 and 15% of the basal diet. Other experimental procedures were the same as described for previous experiment.

*Results.* Addition of lactose to a practical diet containing dibasic sodium phosphate as source of phosphorus had no appreciable effect on either percent bone ash or body weight at 4 weeks of age (Table I). Slight decreases in weight were observed when lactose was added at levels of 5 and 10%, but at the 15% level the weight was equal to that of Group 2 which received dibasic sodium phosphate, with no added lactose.

Groups of chicks fed colloidal phosphate as sole source of phosphorus showed improvement in growth rate with addition of 10 or 15% lactose. Addition of 5% lactose had very little effect on growth rate, but increased availability by 8.3 percentage points when measured in terms of bone ash. Increasing lactose level in the diet containing colloidal

TABLE I. Effect of Addition of Lactose to a Practical-Type Diet on Chick Growth and Bone Formation at End of 4 Weeks, in 9 Groups.

| Lactose level (%) | Added phosphorus (%) | Source | 4 wk wt (g) | Bone ash (%) | Feed conversion | Availability (%)* |
|---|---|---|---|---|---|---|
| 0 | 0 | | 290.8 | 33.58 | 2.49 | |
| 0 | .2 | $Na_2HPO_4$ | 343.1 | 41.83 | 1.97 | |
| 5 | " | | 335.6 | 42.65 | 2.05 | |
| 10 | " | | 314.8 | 42.10 | 2.05 | |
| 15 | " | | 343.3 | 43.30 | 1.90 | |
| 0 | " | Colloidal phosphate | 283.2 | 38.55 | 2.37 | 82.6 |
| 5 | " | | 285.0 | 38.77 | 2.10 | 90.9 |
| 10 | " | | 300.5 | 39.52 | 2.05 | 93.8 |
| 15 | " | | 303.2 | 40.15 | 2.14 | 94.9 |

* Availability relative to that of $Na_2HPO_4$ was determined by comparing bone ash values of groups with corresponding lactose levels when different sources of phosphorus were used.

phosphate to 10% resulted in increased growth as well as bone ash. Groups of birds fed 10% lactose showed an 8.5% increase in growth rate over that of the group fed a diet containing no added lactose. Phosphorus availability, based on comparison of bone ash with that of corresponding groups fed dibasic sodium phosphate, indicated that addition of 10% lactose to the diet increased 11.3 percentage points in availability of phosphorus in colloidal phosphate. Increase of lactose level to 15% had very little effect on rate of growth and percent bone ash, as compared to group fed 10% lactose. Phosphorus availability in this case was 12.4 percentage points greater than that observed in the group fed colloidal phosphate alone.

The pH measurements at different parts of intestine are summarized in Table II. With increases in lactose level of the diet, a regular decrease was observed in pH of duodenum from 6.8 to 6.5. The pH of caecal contents dropped less consistently from 6.3 to 5.4. These data are in general agreement with the findings of Beach *et al.*(9), that with increase of lactose level of the diet an increase in intestinal acidity takes place.

TABLE II. Effect of Lactose on Intestinal pH of Chicks Fed a Practical-Type Diet.

| Added lactose (%) | pH* | | | |
|---|---|---|---|---|
| | Duodenum | 4″ posterior to duodenum | Caecum | 4″ anterior to caecum |
| 0 | 6.8 | 6.5 | 6.3 | 7.0 |
| 5 | 6.7 | 6.3 | 5.8 | 6.8 |
| 10 | 6.6 | 6.4 | 5.4 | 6.4 |
| 15 | 6.5 | 6.3 | 5.7 | 6.1 |

* Avg of 10 values/treatment.

Increased availability of colloidal phosphate (Groups 7, 8 and 9) with increased lactose content of diet may have been due to increased acidity of the intestinal tract. This could create favorable conditions for bringing the colloidal phosphate into a soluble state. Lactose is hydrolyzed and absorbed with difficulty. For this reason, it remains for a considerable length of time in the intestinal tract, and may produce a favorable environment for growth of aciduric types of intestinal flora. An increase in acidity following growth of these aciduric bacteria may cause increased release of phosphorus and calcium from colloidal phosphate, resulting in increased availability of phosphorus to the chicks.

Efficiency of feed conversion improved considerably from 2.37 for birds fed colloidal phosphate alone to 2.10, 2.05 and 2.14 with groups fed colloidal phosphate supplemented with 5, 10 and 15% lactose, respectively (Table I).

Addition of lactose to a purified chick diet depressed growth rate in Groups 2, 3 and 4 fed dibasic sodium phosphate (Table III). Growth of birds receiving phosphorus as equal parts of colloidal phosphate and dibasic sodium phosphate was stimulated by supplementation with lactose (Groups 6, 7, and 8) as compared to Group 5, fed phosphorus from colloidal phosphate and dibasic sodium phosphate with no added lactose. This indicated a favorable effect of lactose on utilization of phosphorus from colloidal phosphate. Relative availability increased from 93 to 101% with increase in lactose level up to 10% of the diet.

TABLE III. Effect of Addition of Lactose to a Purified Diet on Chick Growth and Bone Formation at the End of 4 Weeks.

| Groups and supplements | Added phosphorus (%) | Lactose (%) | 4 wk wt (g) | Bone ash (%) | Availability (%)[*] |
|---|---|---|---|---|---|
| 1. $Na_2HPO_4$ | .4 | 0 | 408.5 | 36.08 | |
| 2. ” | ” | 5 | 378.1 | 35.61 | |
| 3. ” | ” | 10 | 383.3 | 35.48 | |
| 4. ” | ” | 15 | 377.1 | 34.86 | |
| 5. ½″ + ½ colloidal phosphate | ” | 0 | 278.0 | 33.56 | 93 |
| 6. *Idem* | ” | 5 | 362.5 | 35.96 | 101 |
| 7. ” | ” | 10 | 341.9 | 35.84 | 101 |
| 8. ” | ” | 15 | 351.5 | 33.16 | 95 |

[*] Availability relative to that of $Na_2HPO_4$ was determined by comparing bone ash values of groups with corresponding lactose levels when different sources of phosphorus were used.

*Summary.* The effect of lactose, at levels of 5, 10 and 15% of purified and practical diets, on utilization by chicks of phosphorus from colloidal phosphate was studied. Data presented here demonstrated that utilization of colloidal phosphate by chicks was improved when diets were supplemented with lactose at levels to 10%. The pH of intestinal tract was lowered by lactose supplementation, although this effect was not uniform as lactose level increased.

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# Estimation of Cardiac Output by Rapidly Repeated Dye-Dilution Technic.

## (25377)

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A method for estimating cardiac output every 5-8 seconds has been devised. The procedure, based on the indicator dilution principle, has been used in acute experiments where rapidly repeated measurements of cardiac output were required.

*Method.* A small (Intramedic, PE-100) polyethylene catheter was introduced into the left atrium of anesthetized dogs *via* a pulmonary vein. The small intercostal incision in the fourth left interspace was left open and mild artificial respiration was continued throughout the experiment. The catheter was connected to a specially designed injection valve which controlled the injection of the dye-indicator solution into the left atrium. Time between injections and duration of injection could be pre-determined and phased with other events, such as respiration, by means of electronic controls. A schematic diagram of the injection valve is shown in Fig. 1. The dye-indicator was a tricarbocyanine dye (Cardio-Green®)[*] prepared as a 0.1% solution. Approximately 0.5 mg (for 10-12 kg

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[*] The Cardio-Green® dye for this project was supplied generously by Hynson, Westcott and Dunning, Baltimore, Md., through the courtesy of Dr. John H. Brewer.

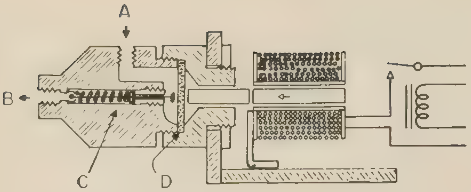

FIG. 1. Dye injection valve. A, inlet from pressurized (600 mm Hg) dye reservoir; B, outlet to dye catheter; C, Standard Schroder tire valve core; D, stiff rubber impregnated fiber diaphragm. Solenoid may be of any type powerful enough to operate valve. Size of assembly is not critical.

dogs) was injected per cycle. Constancy of delivery from the injection valve was established at the beginning of each experiment by collecting in previously weighed vials 10 consecutive deliveries of dye solution. The vials were immediately reweighed before proceeding with the experiment. The results of several typical calibrations are shown in Table I. Arterial blood from an iliac artery (or the descending aorta) was drawn through a Colson densitometer(1) by a specially designed, nonfreezing, constant rate pump. The pump had a capacity of 40 ml which allowed continuous runs to 80 seconds. The densitometer was fitted with appropriate light filters for the dye and its signal output was rcorded on an Electronics for Medicine recorder. The densitometer was calibrated at the end of each experiment by making several known dye dilutions in blood and passing these through the densitometer. Carotid artery pressure was measured by means of a Statham P-23AA strain gauge. Coagulation of blood was prevented by liberal use of heparin.

TABLE I. Calibration of Dye Injection Valve, in 10 Consecutive Deliveries.

| | June 30, '59 | July 6, '59 | July 16, '59 |
|---|---|---|---|
| | Wt in g | | |
| | .5485 | .5540 | .5648 |
| | .5496 | .5498 | .5455 |
| | .5599 | .5475 | .5600 |
| | .5542 | .5491 | .5495 |
| | .5539 | .5498 | .5525 |
| | .5492 | .5571 | .5634 |
| | .5578 | .5523 | .5644 |
| | .5459 | .5406 | .5586 |
| | .5481 | .5445 | .5599 |
| | .5505 | .5455 | .5632 |
| Avg | .5517 | .5490 | .5581 |
| Range | .5459–.5599 | .5406–.5571 | .5455–.5648 |
| Max error | +2.54% | +2.96% | +3.53% |

*Results.* Fig. 2 shows a continuous record of 7 consecutive optical density curves (fifth from top, Fig. 2) during a period of cardiovascular equilibrium. Dye injections were made precisely 4.1 seconds after onset of lung inflation and, thus, always occurred at the same time during the expiratory pause. Onset of injection is marked on the record both by the injection valve signal (broad pip, top trace, Fig. 2) and by increase of pressure in the injection catheter (third trace from top, Fig. 2). Left atrial pressure fluctuations can be seen on the latter trace. Intratracheal pressure, from which constancy of the respiratory cycle (8.3 sec.) can be checked, is recorded on the second trace from the top of Fig. 2.

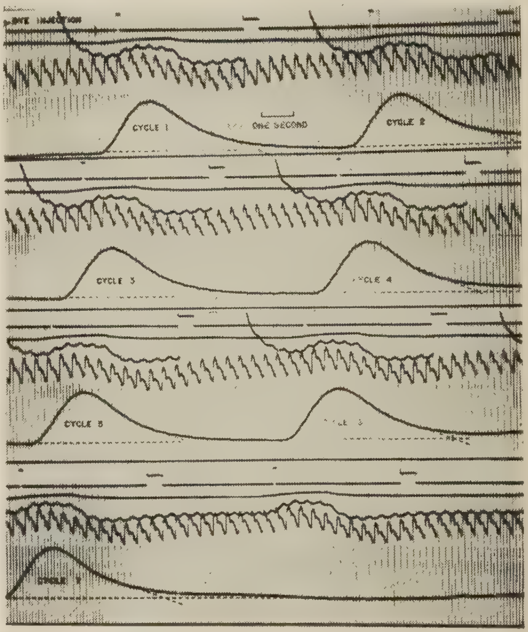

FIG. 2. Continuous record of 7 consecutive dye injections each phased to occur during period of lung deflation in an open chest dog. Top trace, inj. valve signal, narrow pip indicates onset of lung inflation, broad pip marks dye inj. Second trace from top, intratracheal pressure. Third trace, dye inj. catheter pressure (leaves record at moment of inj.). Fourth trace, carotid blood pressure. Fifth trace, dye-dilution curves (continuous, follow to next panel below). Bottom trace, base line.

Due to the relatively small amount of dye injected, and the location of the injection site, recirculation of the dye does not introduce a major difficulty or error in calculation of cardiac output. The major recirculation

TABLE II. Data from 7 Consecutive Dye-Dilution Curves.

| Area ($mm^2$) | Base line (mm) | Mean ht (mm) | Mean conc. ($\gamma$/ml) | Duration of cycle (sec.) | Cardiac output (ml/min.) | Appearance time (sec.) | Foot-peak time (sec) | Peak-ht (mm) |
|---|---|---|---|---|---|---|---|---|
| 2300 | 123 | 18.8 | 6.2 | 5.12 | 1080 | 3.30 | 1.71 | 39.0 |
| 2560 | 135 | 19.0 | 6.3 | 5.63 | 965 | 3.00 | 1.79 | 40.0 |
| 2210 | 121 | 18.3 | 6.2 | 5.05 | 1097 | 3.00 | 1.67 | 38.5 |
| 2450 | 125 | 19.6 | 6.5 | 5.20 | 1050 | 3.25 | 1.67 | 39.5 |
| 2365 | 123 | 19.2 | 6.3 | 5.12 | 1060 | 3.20 | 1.71 | 39.5 |
| 2500 | 130 | 19.3 | 6.4 | 5.40 | 994 | 3.27 | 1.73 | 39.5 |
| 2450 | 132 | 18.6 | 6.0 | 5.50 | 1035 | 3.23 | 1.83 | 40.0 |
| * 2405 | 127 | 18.9 | 6.2 | 5.28 | 1040 | 3.17 | 1.73 | 39.4 |
| † 45.71 | 2.01 | 1.67 | .01 | .26 | 17.67 | .05 | .02 | .20 |

* Mean. † Stand. error.

effect may be seen at extreme right end of cycle 7, Fig. 2. However, the descending limbs of the curves do not reach base line before onset of the following curve due to accumulation of dye in the circulating pool and a "tailing off" of curves which seems to be associated with a binding of the dye to glass surfaces of the densitometer cuvette. For these reasons a base line must be constructed for each curve. The point of abrupt increase in optical density is determined and a base line constructed parallel to the record base line (dashed horizontal lines, Fig. 2). The point at which the descending limb should intersect this base line is determined by plotting the slope of the descending limb against time on semilogarithmic paper (see dashed extrapolation lines, Fig. 2) according to the method of Hamilton(2,3). Areas under the curves were measured with a planimeter and the calculation of cardiac outputs made by the classical Stewart formula.

Table II presents results of calculations made on the record shown in Fig. 2. Data on several parameters, in addition to cardiac output, are included for the benefit of those interested in the more theoretical aspects of indicator dilution curves.

*Discussion.* Under conditions of cardiovascular equilibrium reproducibility of these consecutive dye-dilution curves is impressive. More so, because it was thought probable that mixing of the dye in the left atrium and ventricle would be incomplete. It seems unlikely that results as remarkably consistent as those shown in Table II could be obtained routinely if this is the case. Data from 30 experiments indicate that variation of calculated cardiac output under conditions of cardiovascular equilibrium is no more than ± 5% which is just about the magnitude of inherent error of the instruments used.

Some precautions are necessary to obtain consistent results. Most important is the necessity of phasing the dye injection with some point in the respiratory cycle, since cardiac output may vary as much as 30% during the respiratory cycle. In 8 experiments cardiac output averaged 20.8% less when the dye injection was made during lung inflation than when the injection was made during the expiratory pause (lung deflated).

This application of the indicator-dilution principle is made possible, of course, by the characteristics of the tricarbocyanine dye(4). Its rapid clearance permits numerous injections without reaching a point of optical saturation. Although the disappearance curve of the dye is steep, no correction has been necessary for continuous runs of less than 80 seconds involving a total injection of about 9 mg of dye. Light transmission characteristics of the dye make fluctuations of arterial oxygen saturation unimportant and permit application of the method to experiments involving asphyxia or marked respiratory changes.

Since quantitative accuracy of the method has not been checked by doing simultaneous Fick determinations, it is not known how the 2 determinations would compare. However, cardiac outputs as measured by the rapidly repeated injection technic are within expected ranges and change in the expected direction and to the expected degree when conditions are changed.

*Summary.* Injection of 0.5 mg of a tricarbocyanine dye into the left atrium of anesthetized dogs every 5-8 sec. (phased with respiration) results in reproducible dye-dilution curves during cardiovascular equilibrium. The calculated cardiac output does not vary from determination to determination more than ± 5%. The method is useful in the investigation of problems requiring rapidly repeated estimations of cardiac output.

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## Metabolism of Aminonitriles and Related Compounds by the Rat.* (25378)

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During our studies on metabolism of β-amino-propionitrile (BAPN), the rat lathyrism factor, considerable variation in severity of symptoms was observed among individual animals(1). The rat eliminated unchanged BAPN in the urine and also a metabolite identified as cyanoacetic acid (CAA)(2). Since the latter compound produced none of the symptoms of BAPN toxicity when fed to weanling rats(3), it presumably is a detoxification product. Using a method for quantitative determination of CAA(4), a study has been carried out on relationship between efficiency of conversion of BAPN into urinary CAA and severity of skeletal deformities. The metabolism of a number of organic nitriles related to BAPN has also been investigated to determine whether other nitriles are likewise detoxified by conversion into CAA. A preliminary account of part of the latter work has been reported(5).

*Methods and materials.* BAPN was used as the crystalline fumaric acid salt (2 BAPN · 1 fumaric acid), aminoacetonitrile as the hydrogen sulfate acid salt, and 2,2′-iminodipropionitrile (IDPN) as the free base.† The aminonitrile analogs, 4-aminobutyronitrile hydrochloride (mp 144.5-145.5°) and 5-aminovaleronitrile hydrochloride (mp 121-122.5°) were synthesized from corresponding chloronitriles by procedure of Goldberg and Kelley (6). Analysis of 4-aminobutyronitrile hydrochloride. Calc'd for $C_4H_9N_2Cl$: N, 23.23; Cl, 29.40. Found: N, 23.50; Cl, 29.50. Analysis of 5-aminovaleronitrile hydrochloride. Calc'd. for $C_5H_{11}N_2Cl$: N, 20.81; Cl, 26.34. Found: N, 20.84; Cl, 26.70. The intermediate 4-phthalimidobutyronitrile melted at 79-81° instead of 65-66° as reported(6) and the intermediate 5-phthalimidovaleronitrile had mp 77.5-79°. Analysis of 4-phthalimidobutyronitrile. Calc'd. for $C_{12}H_{10}O_2N_2$: N, 13.09. Found: N, 13.90. Analysis of 5-phthalimidovaleronitrile. Calc'd. for $C_{13}H_{12}O_2N_2$: N, 12.27. Found: N, 12.62. Propionitrile, n-butyronitrile, n-valeronitrile, n-hexanenitrile, succinonitrile, and CAA were Eastman products. Ethylene cyanohydrin was furnished by American Cyanamid Co. *Animal experiments.* Sprague-Dawley female rats were used for studies of relationship between urinary CAA levels and severity of skeletal deformities. Older rats weighing 90-300 g were used for study of metabolism of various organic nitriles related to BAPN. The weanling rats (6/group) were fed one of 3 basal diets which differed only in their protein composition (see Table I). Test diets corresponding to the 3 basals were prepared by addition of 3 g of BAPN fumarate to 1 kg of diet. Feeding was *ad lib.* except during

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† These compounds were kindly supplied by Abbott Labs., North Chicago, Ill.

TABLE I. Basal Diet A.*

| Ingredient | Amt |
|---|---|
| Casein (Borden's), ''vitamin-free'' | 120 g |
| Cerelose (dextrose) | 780 " |
| Corn oil | 60 " |
| Salts (Wesson's) | 40 " |
| Choline chloride | 1 " |
| DL-Methionine | 1 " |
| L-Cystine | 1 " |
| Thiamine • HCl | 25 mg |
| Riboflavin | 3 " |
| Pyridoxine • HCl | 25 " |
| Calcium pantothenate | 20 " |
| Nicotinic acid | 10 " |
| i-Inositol | 10 " |
| Biotin | .5 " |
| Folic acid | 2 " |
| Menadione | 10 " |
| Vitamin A acetate | 6000 IU |
| " D | 60 " |
| α-Tocopherol | 100 mg |

* Basal diets A, B, and C were alike except that diet B had crude instead of ''vitamin-free'' casein, and diet C had 240 g crude casein and 660 g cerelose. All minor components were mixed into the dry mixture, except for fat-soluble vitamins which were blended into corn oil before mixing with dry ingredients.

collection of urine. Older rats were maintained on commercial Purina pellet diet. Nitriles were injected into these animals intraperitoneally as neutral aqueous solutions or suspensions of 1-2 ml volume, equivalent to dose of 20-50 mg/rat. *For collection of urine* rats were placed for 18 hours in wire metabolism cages over large glass funnels. A glass baffle at funnel outlet and over the bottle receiving urine, deflected the solid feces and thus minimized contamination. Urine was collected from weanling rats after they had been kept on control or test diets for 5 weeks. In rats to which organic nitriles were administered, urine collection was made immediately following injection of the chemical being studied. During collection period the animals were given water *ad lib.* but no food. At end of collection period the funnels were rinsed with a few ml of water, and the combined urine and wash water filtered through glass wool. The slightly diluted urine samples so obtained were then examined for CAA content either by the quantitative method(4), or qualitatively by chromatographing on paper as previously described(2). In the latter case duplicate chromatograms were sometimes prepared and sprayed with 0.1% ferric chloride, 3% hydrogen peroxide reagent to detect thiocyanate(7). *Colorimetric assay for CAA of urine from rat receiving radioactive BAPN.* Urine was collected from a rat injected with C$^{14}$-cyano labeled BAPN(2). Parallel radioactivity and CAA measurements were carried out on an ethyl acetate extract of acidified urine. By the colorimetric assay a value of 2.50 $\mu$M of CAA/ml of urine was found. Calculations based on radioactivity of the extract and the known specific activity of administered BAPN showed that 2.63 $\mu$M of CAA could have been present. The close agreement indicated that the ethyl acetate extract of acidified urine contained radioactivity mainly in the form of CAA. *Analysis of urine for CAA.* A 1 ml volume of urine, acidified with 0.1 ml of 1:1 (v/v) sulfuric acid:water, was extracted 6 times with 2 ml portions of ethyl acetate in 6″ test tube or 15 ml centrifuge tube. After separation of layers by settling or centrifuging, the ethyl acetate layer was withdrawn using a glass tube with drawn-out capillary tip. The combined ethyl acetate extracts were washed twice with 0.2 ml of water, then evaporated to dryness in 25 ml beaker in the draft of laboratory hood. The residue was dissolved in a small measured volume of distilled water and used for quantitative colorimetric determination of CAA as previously described(4). *Determination of skeletal deformities.* Animals on BAPN and basal diets were examined for skeletal deformities by manual palpation, primarily noting the everted sterna. Estimations of severity of deformities were expressed numerically as follows: normal, 0; minimal, 1; moderate, 2; severe, 3. Rats with grade 0, 1, or 3 sternal deformities were selected from different groups for urine collection and urinary CAA analysis. At termination of experiment, postmortem examinations were performed on all animals and deformity ratings as estimated on living animals were verified or modified, by visual inspection of skeletal structures.

*Results. Urinary CAA and its relationship to severity of skeletal deformities.* In Table II results on relationship between quantity of urinary CAA and severity of bony deformities in rat lathyrism are presented. Urinary CAA

TABLE II. Relationship between Urinary CAA in Rat Lathyrism and Severity of Bony Deformities.

| Rat No. | Diet | Supplement | Time on diet (days) | Rat wt (g) | Urinary CAA (mg/18 hr)* | Severity of bony deformities |
|---|---|---|---|---|---|---|
| M- 1 | Basal A | None | 37 | 135 | .17 | 0 |
| 1 | " | " | 39 | 139 | .17 | 0 |
| 1 | " | " | 49 | 164 | .24 | 0 |
| 4 | " | " | 51 | 157 | .09 | 0 |
| 16 | " | .3% BAPN | 39 | 120 | 1.10 | 1 |
| 16 | " | " | 49 | 130 | .76 | 3 |
| 10 | " | " | 51 | 123 | .91 | 1 |
| 21 | Basal B | None | 35 | 128 | .08 | 0 |
| 33 | " | .3% BAPN | 37 | 96 | .73 | 3 |
| 33 | " | " | 47 | 106 | .62 | 3 |
| 33 | " | " | 49 | 106 | .67 | 3 |
| 32 | " | " | 39 | 101 | .70 | 3 |
| 32 | " | " | 49 | 112 | .63 | 3 |
| 34 | " | " | 37 | 76 | .31 | 3 |
| 35 | " | " | 37 | 105 | 1.18 | 1 |
| D- 1 | Basal C | None | 49 | 166 | .20 | 0 |
| 13 | " | .3% BAPN | 49 | 138 | 1.95 | 1 |
| 15 | " | " | 49 | 129 | 2.38 | 1 |
| 16 | " | " | 49 | 135 | 1.70 | 1 |

* Urine from rats on unsupplemented basal diets contained no CAA; the values shown in this table represent "apparent CAA" due to interfering substances present in normal rat urine (see text).

values are given as total amount excreted in 18 hours. In urines from normal rats on basal diets A, B and C, not receiving BAPN, the observed absorbancy values were calculated as "apparent CAA," and varied from 0.08 to 0.24 mg/18 hour urine sample. Since examination of extracts of such normal rat urines on paper chromatograms failed to indicate presence of CAA, these values represent interfering substances. CAA values of test urine samples were not corrected for this "apparent CAA."

Range of CAA excretion values for rats with minimal (grade 1) bony deformities was 0.91 to 1.18 mg on the 12% protein diets and 1.70-2.38 mg on 24% protein diet. For rats with maximal (grade 3) osseous changes, observed in these experiments on the 12% casein diets, urinary CAA excretions were definitely lower, being 0.62-0.76 in all cases except one which was 0.31 mg. Animals with moderate (grade 2) bony changes were not studied.

The higher urinary CAA excretion by rats with minimal osseous deformities suggests that these animals were better able to detoxify BAPN by converting it into CAA. A quantitative study of urinary excretion of BAPN by rats having symptoms of varying severity would be an interesting supplement to this work. The higher CAA excretion of rats on 24% as compared with 12% casein diet, and the associated reduction of severity of the symptoms, support the previous observations of Dasler(8) that high levels of casein exert a protective effect.

*Excretion of CAA following administration of organic nitriles.* Tables III and IV present data on urinary CAA excretion following intraperitoneal injection of various organic nitriles related to BAPN. The qualitative results of Table III were obtained by chromatographing ethyl acetate extracts of the various urines on paper(2). Nitriles, besides BAPN, which were thus found to give rise to CAA *in vivo* included IDPN, ethylene cyanohydrin, succinonitrile, 5-aminovaleronitrile, and n-valeronitrile. Traces of CAA were also detected in urines of some animals which received n-hexanenitrile and 4-aminobutyronitrile, although previously CAA was not observed following injection of the latter(5).

Quantitative determinations in Table IV were made subsequently to confirm and extend the qualitative results. As emphasized above, interfering substances in normal rat urine result in a blank, "apparent CAA" value, the approximate magnitude of which may be judged from data on control rats.

TABLE III. Urinary Cyanoacetic Acid following Injection of Various Aliphatic Nitriles in Rats.

| Compound | Dosage of nitrile/rat mg | Dosage of nitrile/rat $\mu$M | Approx. wt of rats* (g) | Intensity of CAA zone in chromatogram of urine | Remarks |
|---|---|---|---|---|---|
| Aminoacetonitrile • HCl | 30 | 320 | 300 | 0 | |
| BAPN • HCl | " | 280 | 100 | +++ | |
| 4-Aminobutyronitrile • HCl | 20, 42 | 170, 350 | 150, 300 | 0, 0 | |
| " | 30, 50 | 250, 420 | 100, 290 | tr, tr | |
| 5-Aminovaleronitrile • HCl | ", " | 220, 370 | ", 300 | tr, + | |
| N-Propionitrile | 50 | 910 | 180 | | Animal died after inj. |
| N-Butyronitrile | 20, 33 | 290, 480 | 100, 160 | ---, 0 | 100 g animal died overnight |
| N-Valeronitrile | 33, 50 | 400, 600 | ", 200 | +, + | *Idem* |
| " | 20 | 240 | 150 | + | |
| N-Hexanenitrile | ", 50 | 210, 520 | ", 175 | 0, tr | 175 g animal died |
| IDPN | " | 160 | 140 | +++ | |
| Ethylene cyanohydrin | " | 280 | 300 | +++ | |
| Succinonitrile | 15† | 190 | " | + | |

* All except marked animals (+) are female. † Given in drinking water. Approx. dose.

Another source of error was inefficiency of extraction procedure as applied to urine. Determinations of recovery of 50 $\mu$g quantities of CAA added to 1 ml of normal rat urine indicated that only 75% of the CAA was recovered. These 2 errors would tend to counterbalance each other to some extent, but no effort has been made to apply any correction factor to values shown in Table IV.

Expressed as mole % of administered nitrile, urinary CAA values following injection of BAPN and IDPN were 11.1 and 15.2, respectively. The excreted CAA resulting from 4 to 6 carbon alkyl- and amino-nitriles accounted for 2% or less of administered nitriles. Although CAA apparently represents only a minor metabolic product of 4 to 6 carbon nitriles related to BAPN, its appearance in urine demonstrates that both amino- and alkyl-nitriles undergo some degradation from the end of the molecule opposite the cyano group.

Early studies on nitrile metabolism have emphasized direct attack on the nitrile group (9) with conversion either to a carboxyl group, or to thiocyanate. Although we observed traces of thiocyanate ($R_f$ *ca* 0.25 in a butanol:acetic acid:water system, 4:1:1 by volume) in all rat urines, including normals, no marked increase appeared on chromatograms corresponding to any particular nitrile. The major form of excretion of most of the

TABLE IV. Quantitative Determination of Nitrile Excreted in Urine as Cyanoacetic Acid by the Rat.

| Compound | Dosage ($\mu$M/animal) | Wt of animal (g) | Urinary CAA* ($\mu$M after 18 hr) | Fraction recovered as CAA (mole %) avg | Intensity of CAA zone on chromatograms |
|---|---|---|---|---|---|
| BAPN | 230 | 93, 105 | 51.8 | 11.2 | +++ |
| 4-Aminobutyronitrile • HCl | 250 | 89, 91 | 2.9 | .58 | — |
| 5-Aminovaleronitrile • HCl | 220 | 96, 96 | 4.4 | 1.0 | + |
| N-Butyronitrile | 300 | 153, 162 | 1.1 | .18 | — |
| N-Valeronitrile | 245 | 148, 146 | 7.4, 11.9 | 2.0 | + |
| N-Hexanenitrile | 205 | 144, " | 2.0, 3.5 | .67 | — |
| IDPN | 160 | 135, 144 | 51.1, 47.3 | 15.4 | +++ |
| None (control) | 0 | 128, 164 | .9, 3.8 | | — |

* Correction for apparent CAA of normal rat urine has not been made. Where one figure is given it represents avg of 2 values in close agreement. In other cases, the 2 values represent individual determinations. In each case, however, determinations were made on pooled urine from the 2 rats used for each compound, and CAA excretion values are totals for both animals.

nitriles is not disclosed by present studies. Unchanged nitriles and ω-carboxy-nitriles which have undergone no degradation of the carbon chain may represent the major types of urinary excretion products. Identification of α-cyanopropionic acid and unchanged 2-cyanopropylamine as urinary excretion products of 2-cyanopropylamine have been reported(10).

*Summary.* 1. Among weanling rats receiving 0.3% dietary BAPN fumarate those showing less severe skeletal deformities tended to excrete relatively larger amounts of cyanoacetic acid (CAA) in urine, and vice versa. Increasing dietary casein level from 12 to 24% resulted in less severe skeletal lesions and higher urinary CAA excretion for a given dosage of BAPN. 2. Appreciable urinary excretion of CAA followed injection of the following organic nitriles into rats: 2,2′-iminodipropionitrile (IDPN), ethylene cyanohydrin, succinonitrile, 5-aminovaleronitrile, and n-valeronitrile. Traces of CAA resulted from injection of n-hexanenitrile and 4-aminobutyronitrile.

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## Development of Resistance by Cell Cultures to Photodynamic Action.
(25379)

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During studies of poliovirus by plaque assay technic(1) it was observed that the uppermost layer of monkey kidney (MK) culture would frequently degenerate within 48 hours after application of overlay medium. The present study was undertaken to determine which factors controlled this cell degeneration.

*Materials and methods. Cell cultures.* Monolayer cultures of MK(2) in 2 oz. bottles were grown in Medium 199 containing 2% calf serum.* *Media.* Four preparations of Medium 199(3) were employed in these experiments—Medium 199 containing no dye, Medium 199 containing 1:400,000 phenol red, plaque Medium 199 containing no dye, and plaque Medium 199 containing 1:50,000 neutral red. *Light source.* In most experiments a fluorescent desk lamp containing two 15 watt daylight-type tubes was placed 9 inches above the cell cultures. Light intensities were measured by a Weston light meter model 715. *Extraction of neutral red from cells.* MK cultures in 2 oz. bottles were fed 5 ml fluid Medium 199 containing 1:50,000 neutral red and incubated in the dark at 36°C for 48 hours. Each culture was rinsed 3 times with BSS, placed in contact with 1.0 ml of 2-fold concentrated Medium 199, frozen and thawed twice and incubated at 36°C for 4 hours. Cell debris was removed by low speed centrifugation and the clarified plaque medium mixed with agar before application to fresh cultures.

*Results.* To determine whether degeneration of the uppermost layer of MK cultures resulted from overheating of top bottles by sunlight which entered the laboratory window, thermometers were placed on each of the 5 shelves of metal racks both within and beside culture bottles. Temperatures were recorded every 5 minutes during the routine performance of a plaque neutralization test. This work was done at a laboratory table in front of a window in intermittent sunlight. The highest temperature recorded was 36°C (on the top shelf) which is the temperature at which these tests are usually incubated. Although temperature was never excessive, MK cultures that were on the top shelf showed marked degeneration by the next day. Since excess heat did not cause degeneration of these cells, effect of room light was investigated next. Twenty MK cultures were overlayed with Medium 199 containing 1:50,000 neutral red and exposed to ambient light in the laboratory at 10 minute intervals over a 120 minute period. Cultures were then incubated for 24 hours and observed for degeneration. A 10 minute exposure resulted in no degeneration; a 30 minute exposure resulted in 75% cell destruction and thereafter destruction of the cell sheet was complete, indicating that light was one of the factors which caused cell degeneration. Light meter readings of incident light varied from 75 to 400 foot candles during the experiment. In subsequent experiments cultures were exposed to a fluorescent light source at an illumination of 100 foot candles at the location of the culture bottles.

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* The cultures were prepared by the cell culture laboratory of Division of Biologics Standards, N.I.H. under supervision of Dr. Paul Gerber.

An experiment was designed to determine (a) minimum exposure time required for complete cell destruction and (b) whether neutral red was required for cell damage. Six MK cultures were overlaid with plaque medium containing neutral red; 6 MK cultures were overlaid with plaque medium without neutral red. A control area of cells was included in each culture by placing opaque masking tape over the lower portion of each bottle. Two cultures with neutral red and 2 without neutral red were exposed to light for 5 minutes. Similar groups of 4 cultures each were exposed for 15 and 30 minute periods. Cultures which were overlaid with medium containing no neutral red did not degenerate after exposure to light, while cultures overlaid with medium containing neutral red showed partial degeneration after 15 minutes exposure and complete destruction after 30 minutes exposure.

Similar results were obtained with plaque medium without protein or containing 2% calf serum or 20% skim milk(4). HeLa(5), HEP 2(6), and continuous human amnion(7) cultures also gave similar results. Fluid medium (Medium 199 or basal medium Eagle) (8) containing neutral red similarly sensitized MK cultures to light while fluid medium without indicator or with phenol red (1:400,000) did not sensitize cultures.

It was then observed that control areas of MK culture bottles (shielded from light by masking tape) which had been stained with neutral red for 2 to 10 days did not degenerate after exposure to laboratory illumination. This observation indicated that the cells did not retain sensitization to light over prolonged periods although they were still stained with neutral red. To determine duration of sensitivity to light after application of neutral red MK cultures were ovarlaid with plaque medium containing neutral red, incubated in the dark at 36°C for various intervals (0.5, 1, 2, 4, 6, 8, 12, 16, 24, and 48 hours) and exposed to light in groups of 2 for 30 minutes. Fig. 1 shows some of these cultures. The lower portion of each bottle was shielded from light and served as control area. Cell destruction became increasingly severe up to 1 hour; between 1 and 6 hours destruction was complete; between 8 and 16 hours cell destruction diminished and after 24 hours the cells were resistant to the action of light. The resistance of cells after incubation with neutral red for 24 hours or longer might be due to a change of cells or to alteration of neutral red. To distinguish between these 2 possible mechanisms 4 MK cultures were overlaid with medium containing neutral red and 4 MK cultures were overlaid with medium containing no dye. After incubation in the dark at 36°C for 48 hours (sufficient time for development of resistance) 0.2 ml of a 1:2000 solution of neutral red was added to half the cultures of each group (each culture contained 5 ml of plaque medium). To allow neutral red to penetrate the cells the cultures were incubated at 36°C for 4 hours and then exposed to light

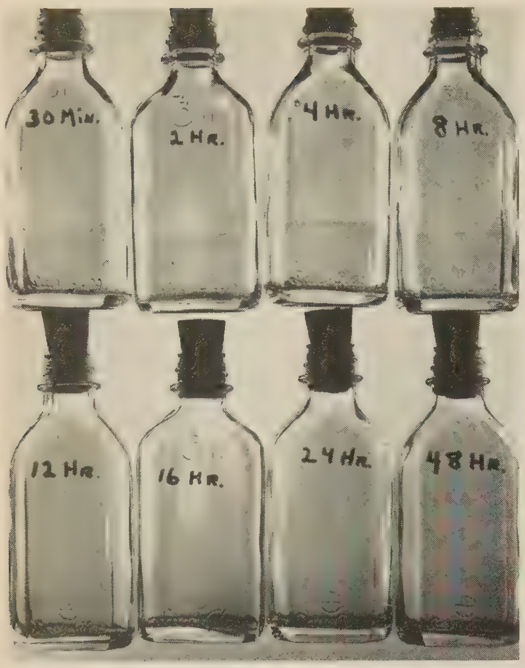

FIG. 1. Photosensitization of MK cultures after preincubation with neutral red for varying periods.

for 30 minutes. Cultures which had developed resistance to light after incubation with neutral red for 48 hours were also resistant to sensitizing action of additional neutral red. Cultures which were incubated for 48 hours in the absence of neutral red were sensitized by addition of neutral red. Control cultures containing a double concentration of neutral red were sensitive to light. It was concluded that loss of sensitization was probably due to a change in the cells rather than alteration of the absorbed neutral red. To determine whether the cell-absorbed neutral red was altered in its ability to sensitize cells, neutral red was extracted from MK cultures which had lost sensitivity to light after incubation with neutral red for 48 hours. Extracted neutral red was applied to fresh MK cultures in plaque overlay and the cultures incubated in the dark at 36°C for 2 hours before exposure to light for 30 minutes. Complete cell destruction was observed 24 hours later, suggesting no alteration of dye.

*Discussion.* The unexpected degeneration of the uppermost layer of MK cultures during plaque assay of viruses was shown to be caused by neutral red and light which acted together to cause cell destruction. Similar degeneration has been observed independently by others(9) and has been demonstrated to be due to neutral red and light(9).

Other investigators have observed damaging effect of vital stains on living cells in the presence of light(10,11,12). The phenomenon of dye-sensitization of living organisms to light has been termed photodynamic action (10). Evidence obtained with microorganisms indicates that dye binds to the organisms, adsorbs light energy and results in a damaging oxidative reaction(10). It is likely that many of the unexplained cell failures during plaque assay may have been due to photodynamic action since there is sufficient illumination in a well lighted laboratory to cause cell destruction after a 30 minute exposure. Damaging effect on cultures used for plaque assay may be avoided if the cultures are placed in the dark within a few minutes of application of overlay medium and not exposed to bright light until 24 hours have elapsed.

The sensitization of cells by neutral red was shown to increase to a maximum at 1 hour after application of overlay medium. Loss of sensitization began at 8 hours and cells were completely resistant to light at 24 hours. Initial increase in sensitization during the first hour may be analogous to a time-dependent sensitization of bacteriophage T2 by toluidine blue(13). To our knowledge, loss of sensitization to photodynamic action has not been reported previously. Results indicate that loss of sensitization was probably due to a change in the cells rather than alteration of absorbed neutral red. One of the possible explanations for development of cellular resistance is an adaptive-like change of the cells(14). Further studies are required to determine the correct explanation.

*Summary.* Unexpected degeneration of uppermost layer of cell cultures was shown to be caused by sensitization of cells by neutral red to damage by light *i.e.*, photodynamic action. Sensitization of cells by neutral red was shown to increase to a maximum at 1 hour. Loss of sensitization began at 8 hours and cells were completely resistant to light at 24 hours. The results indicate that loss of

sensitization was probably due to a change in cells rather than alteration of absorbed neutral red.

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## Utilization and Toxicity of Dialdehyde- and Dicarboxyl-Starches.* (25380)

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The modified physical and taste characteristics of oxidized starch have suggested the possibility of use in foods. A preliminary study of effects on the rat is presented here. Anhydroglucose units of starch can be oxidized by periodic acid to dialdehydes(1). The reaction is stoichiometric so that the number of glucose units thus oxidized can be regulated by amount of periodic acid used. An electrolytic procedure which regenerates the periodic acid as it is used up has recently been introduced(2). Further oxidation by chlorous acid quantitatively converts the aldehyde groups to carboxyl groups(3). The reactions are represented in Fig. 1.

*Materials.* Oxidized cornstarches were obtained from Northern Regional Research Lab., Peoria, Ill. Four dialdehyde starches had 0.05, 2, 40 and 100% of glucose units oxidized. These will be referred to as DAS-.05, -2, -40, and -100, respectively. The dicarboxyl starch had 5% of the glucose units oxidized (DCS-5). *Methods.* *Acute toxicity* tests were not performed with DCS, because some animals in the subacute study were ingesting daily 16-17 g/kg without apparent ill effects. Acute oral toxicity was studied with DAS-100. The material was dissolved with a minimum of NaOH, neutralized immediately with HCl and given to young adult rats (2 males and 5 females) at 1 g/kg. One male rat died after 11 days with multiple lung abscesses, presumably not due to the material. The others remained alive and healthy during 2 weeks observation. The basal diet for subacute toxicity experiments consisted of corn meal, 51.5; casein, 11.5; linseed oil cake meal, 10; alfalfa meal, 2; cod liver oil, 3; bone ash, 1.5; NaCl, 0.5; and cornstarch, 20%. Experimental diets were made by replacing an equal amount of corn starch with the desired amount of the appropriate oxidized starch. The animals were 30-day-old male rats, except for the DAS-0.05 test, where females were used. They were caged in groups of 4 to 6 animals, with water and food available *ad lib.* Feeding of diets continued for 90 to 100 days except when marked toxicity or limited supply forced earlier termination (lack of sufficient DAS-0.05 ended the feeding of 20% diet after 41 days). Concentrations of oxidized starches ranged from 1 to 20% of the diet for each of the DAS products, and from 0.2 to 20% for DCS-5.

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* Histopathological examinations were done by Dr. Wm. E. Ribelin.

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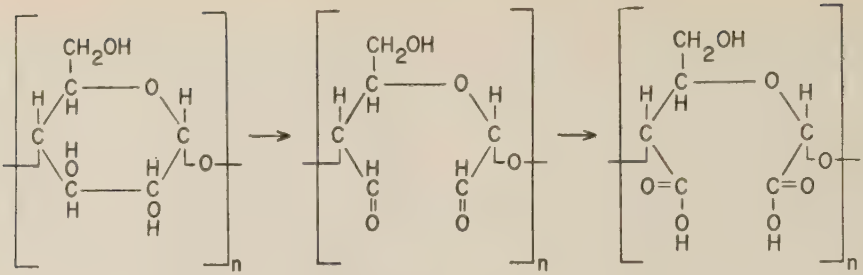

FIG. 1. Oxidation of anhydroglucose units of starch.

*Results.* Continued feeding of diets containing 10 and 20% of DAS-100 led to early death. Concentrations of 5 and 2%, and probably 1%, inhibited growth. DAS-40 permitted normal growth at dietary level of 1%, inhibited growth at 5%, very poor growth at 10%, and early death at 20%. Feeding of DAS-2 and -0.05 permitted normal growth at all levels including 20%. Low food intake accompanied poor growth; in no case was excessive food intake noted. Growth and appearance were normal with DCS-5, but food intake seemed excessive. This will be discussed below.

No gross abnormalities which could be attributed to the diets, were seen at autopsy. Histopathological examination of tissues showed testicular atrophy in rats receiving DAS-100 and -40. In spite of finding testicular atrophy in one control, it appears very probable that DAS-100 produces this atrophy in sexually mature rats in concentrations of 1% or more of the diet. There was an unusual multinucleated cell formation within testicular tubules. Diets containing DAS-40 produced various degrees of testicular atrophy in sexually mature rats in concentrations of 5% or more. Formation of multinucleated cells was not as conspicuous in this group. No significant microscopic abnormalities were detected in any of the DCS-5 animals.

Although growth rates were uniform between groups of DCS animals, there was from the start an apparently much greater food intake for rats receiving the 20% diet than for those on control diet (13.2 g vs 11.2 g/rat/day at the end of experiment), and possibly rats receiving the 2% diet ate more (11.7 g). To check on the possibility of food wastage, the following experiment was tried. Six male rats 90 days old were placed in individual false-bottom cages, with facilities for collection of feces and wasted food. Half received control diet and half the 20% DCS-5 diet. Food wastage was minimal, showing that rejection of objectionable food was not a factor in the results. Body weights, food intake, and weights of air-dried feces were obtained for individual animals at 3- or 4-day intervals for 2 weeks. Growth was fairly uniform. Food intakes and fecal outputs are shown in the Table. It seems clear that the animals were not utilizing the DCS diet as efficiently as that made with unmodified cornstarch. The greater food intake, however, furnished sufficient calories to maintain normal growth.

This poorer utilization evidenced by greater food intake and fecal output suggested that digestion of oxidized starch might be incomplete. This led to the following qualitative test. Boiled cornstarch and DCS solutions (1%) were mixed with saliva. Aliquots of the digestion mixtures were tested at intervals with dilute I-in-KI and with Benedict's qualitative sugar reagent. Both starches were digested to the point of no color with iodine, although rate of digestion seemed to be faster for cornstarch. Probably the reduction of

TABLE I. Effect of DCS-5 on Food Intake and Fecal Output.

| Diet | | Food intake, g/rat/day | Feces, g/rat/day | Feces × 100 / Food intake |
|---|---|---|---|---|
| Control | | 10.57 | 1.04 | 9.8 |
| | | 11.21 | 1.06 | 9.5 |
| | | 11.86 | 1.09 | 9.2 |
| | Avg | 11.21 | 1.06 | 9.5 |
| DCS-5 | | 13.36 | 1.54 | 11.5 |
| | | 11.29 | 1.26 | 11.2 |
| | | 13.14 | 1.56 | 11.9 |
| | Avg | 12.60 | 1.45 | 11.5 |

Benedict's reagent was faster and perhaps more complete with the cornstarch-saliva mixture.

*Summary.* When 40% or more of anhydroglucose units of starch are oxidized to dialdehydes, the material is toxic to rats, as manifested by poor growth, increased mortality and testicular damage. No acute toxicity was noted. No toxicity occurred when 2% or less of the units were oxidized, even at levels of 20% of diet. Starch further oxidized to contain 5% of glucose units in the dicarboxyl form, produced no toxic symptoms with as much as 20% of diet consisting of this modified starch, but food intake was increased and utilization decreased, perhaps due to faulty digestion of the dicarboxyl starch.

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# Biopotency of Non-Tocopherol Compounds in Vit. E Deficiency Diseases.* (25381)

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Recent isolation of several naturally occurring isoprenoid compounds bearing structural resemblances to tocopherols has raised the question of their possible Vit. E activity for animals. These include ubiquinone, a redox compound present in mitochondria necessary for succinoxidase activity(1), its hydroquinone, and solanachromene, a high molecular weight phenol isolated from tobacco(2) structurally related to α-tocopherol. In the present studies the biological activity of these substances, as well as that of acid reduction product of ubiquinone, has been evaluated in relation to muscular dystrophy in the rabbit. In addition, curative activity of the synthetic antioxidant N,N'-diphenyl-p-phenylenediamine (DPPD) with respect to hypercholesterolemia and impaired stability of red blood cells in Vit. E deficiency has been tested.

*Procedure.* New Zealand white rabbits weighing about 1200 g were depleted of Vit. E using the following diet (%): Labco casein 15, sucrose 37.9, corn starch 36.5, molecular-distilled lard 3, cod liver oil 3, salts 446(3) 4, choline chloride 0.1, vitamin premix in glucose(4) 0.5. All treatments were evaluated by curative assay when the first definite signs of muscular dystrophy were discernible by the righting reaction. At least 3 rabbits were used/treatment. The influence of DPPD on hypercholesterolemia associated with Vit. E deficiency(5) was studied in time series by sacrificing animals at 0, 8, 15 and 22 days after onset of treatment. Free plasma cholesterol was determined by the method of Nieft and Deuel(6). The unesterified cholesterol content of skeletal thigh muscle was also determined after grinding the samples with anhydrous sodium sulfate and acid-washed sand, and extracting for 48 hr with skelly-solve F in a Soxhlet apparatus. The hemolytic behavior in hydrogen peroxide of red blood cells from deficient and DPPD-treated animals was determined using modification of procedure of György *et al.*(7). DPPD was administered orally 20 mg daily as a suspension in 1 ml glycerin containing 0.1 ml Tween 80. One group received 35 mg dl-α-tocopheryl acetate in the same carrier 3 times weekly throughout experiment. Negative controls were treated with the carrier alone. Solanachromene was administered in 50 mg amounts daily, orally or intraperitoneally, as a suspension containing 20 parts ethanol and 80 parts 1% aqueous Tween 80. Ubiquinone, its hydroquinone and

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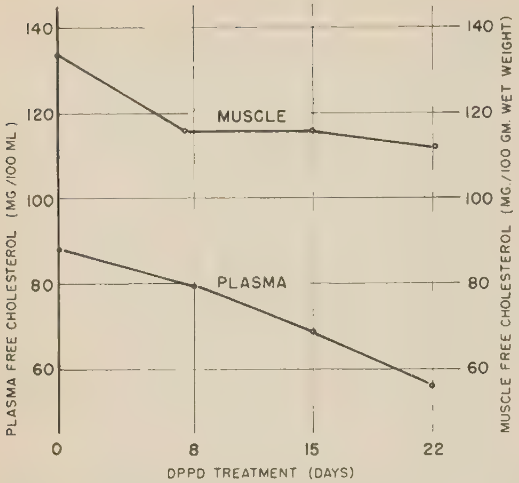

FIG. 1. Influence of DPPD administration on free cholesterol level in plasma and muscle of vitamin E deficient rabbits.

acid-reduction product were prepared immediately before use and formulated similarly. Acid-reduced ubiquinone was obtained by refluxing ubiquinone in ethanolic solution in the presence of stannous chloride and HCl until colorless, then extracting with peroxide-free ether after addition of water. The product was sensitive to Emmerie-Engel reagent, gave $R_f$ values in accordance with literature values and exhibited an ultraviolet absorption maximum at 295 mμ. Hydroquinone was prepared by shaking ubiquinone in the presence of potassium borohydride in aqueous alcohol until the solution was colorless, then extracting with cyclohexane. Both reduced compounds were administered intraperitoneally. Solanachromene also was evaluated in a curative fertility assay using rats. Five female rats were depleted of Vit. E as described previously(8), were carried through a preliminary reproductive cycle to establish their sterility, then were remated to normal males. At 3 day intervals beginning one day prior to 5-day mating period, each female was given orally 18.4 mg solanachrome (a total of 128.8 mg) in an emulsion consisting of 10 parts ethanol and 90 parts 16% Tween 80. Control animals were given 5 mg dl-α-tocopheryl acetate daily.

*Results.* As found previously(9), gross symptoms of muscular dystrophy in rabbits responded rapidly and completely to treatment with DPPD. The data in Fig. 1 demonstrate that a gradual remission of hypercholesterolemia occurred during the regenerative period, as evidenced by decline in free plasma cholesterol from 88.5 to 55.7 mg % in 3 weeks. The latter value approaches the 47.6 mg % concentration for controls which received Vit. E throughout experiment, and indicates that a somewhat longer period is required for complete restoration of normal plasma levels. These results are not in conformity with these of Shull, Ershoff and Alfin-Slater(10) regarding the effect of DPPD on hypercholesterolemia of Vit. E deficiency. The cholesterol content of muscle lipid was greatly elevated in dystrophic animals and, although a decline during the period of DPPD treatment is evident, the final value of 112.5 mg/100 g is still in marked excess of 68.5 mg/100 g found for controls. A regenerative period of 3 weeks is obviously inadequate for restoration of normal cholesterol levels in skeletal muscle.

Erythrocytes taken from dystrophic rabbits were resistant to hemolysis by dialuric acid (11) but hemolyzed immediately in hydrogen peroxide. Following DPPD administration for 15 or 22 days, no hemolysis occurred for several hours. The cells were permeable to hydrogen peroxide, as indicated by oxidation of hemoglobin to methemoglobin, but this conversion did not appear to influence susceptibility to hemolysis and was not affected by DPPD.

No evidence of Vit. E activity was obtained from experiments with ubiquinone or acid-reduced ubiquinone. Up to 200 mg of either compound administered for 1 week, failed to influence the course of the disease or delay death. Similar results were obtained with hydroquinone, administered in 2 or 3 daily doses of 100 mg each. Fifty mg solanachromene daily, administered orally for 5 consecutive days or intraperitoneally for 4 days, also elicited no remission of symptoms or delay in mortality. Further, no offspring were obtained from female rats fed this compound as a supplement to Vit. E deficient diet.

Comparison of molecular structure of solanachromene with that of γ-tocopherol (substitution of a chromene for a chromane ring and 9 unsaturated isoprene units in the side chain for 3 saturated isoprenes) is suggestive of similar functional activity. Based on a minimum effective dose of 0.4 mg α-tocopherol/kg/day for remission of dystrophy in rabbits(12) and a biopotency for γ-tocopherol in relation to α-tocopherol of 8%(13), it may be calculated that if solanachromene and γ-tocopherol were equivalent in activity on a molar basis, 15 mg of solanachromene/day should represent a curative dose for animals in this experiment. Since 50 mg/day failed to elicit any response, the activity of solanachromene, if any, is apparently considerably less than one-third of that of γ-tocopherol.

Our experiments with DPPD extend the number of Vit. E deficiency symptoms which already have been demonstrated to yield to this antioxidant. Previous studies(9) have shown that the preventive and curative effect of DPPD on muscular dystrophy in rabbits and sterility in rats is not due to conservation of Vit. E in tissues or in the diet. In only one respect has replacement been found to be quantitatively incomplete, *viz.*, in maintenance of reproduction in the female rat, which is highly susceptible to DPPD intoxication(8). These and other findings, including effectiveness of DPPD in reversing respiratory decline in necrotic liver degeneration when administered intraportally(14) and the reported absence of Vit. E in tissues of normal chicks fed low-fat diets deficient in this vitamin(15), strongly indicate that the tocopherols represent a class of natural antioxygenic compounds which play an important but non-specific role in metabolism.

*Summary.* Curative tests of Vit. E activity of ubiquinone, its hydroquinone and acid reduction product in relation to muscular dystrophy in the rabbit were negative. Similarly, solanachromene exhibited no biological activity with respect to dystrophy in rabbits or sterility in rats. Vit. E-deficient dystrophic rabbits underwent a remission of gross symptoms within approximately 5 days following oral treatment with N,N′-diphenyl-p-phenylenediamine. The elevated plasma concentration of unesterified cholesterol declined to near-normal levels within 3 weeks, while skeletal muscle concentration responded more slowly. The results confirm previous findings indicating that the Vit. E requirement of rabbits and rats represents a need for suitable antioxygenic compounds.

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# Creatine Phosphokinase Activity in Heart and Skeletal Muscle of Foetal Rabbits.* (25382)

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The evidence which places phosphocreatine and creatine phosphokinase as necessary physiological components of the contraction-relaxation system of muscle is equivocal(1). If it is assumed that phosphocreatine and creatine phosphokinase are necessary for functional contractility, then this relationship should be evident in a close correlation between development of contractility and enzymatic activity in developing foetal muscle. The present investigation suggests that such a close correlation exists in skeletal muscle, but not in heart muscle.

*Methods.* White New Zealand rabbits purchased from local supplier were used. The rabbits were bred while under observation and foetal ages were calculated from this time. At desired time, animals were given an intravenous injection of sodium pentobarbital (40 mg/kg). The foetuses were removed and placed in cracked ice. The hearts and gastrocnemii were removed from 6 to 10 foetuses and pooled. The tissue was removed under a dissecting scope when necessary (14 and 16 day foetuses). Foetal tissue was collected for study only from those rabbits which had from 6 to 10 foetuses. In several instances only 1, 2 or 3 foetuses were present. They appeared larger than usual, and were discarded as well as foetal tissue from rabbits with more than 10 foetuses. The method for creatine phosphokinase extraction and activity determination has been described(2). Creatine and phosphocreatine were determined by the method of Ennor and Rosenberg(3).

*Results.* Creatine phosphokinase activities of heart and skeletal muscle during foetal development are summarized in Table I. The enzyme was not detected in heart or skeletal muscle at 14 days of foetal development. Enzymatic activity was first detected in heart extracts on 16th gestational day, when no creatine phosphokinase could be detected in skeletal muscle. At 20th day of foetal development, enzymatic activity of heart tissue increased approximately 5 times, but was not demonstrable in skeletal muscle at this late stage of development. Creatine phosphokinase activity was first detected in skeletal muscle on the 23rd day of gestation.

The original assumption was that appearance of creatine phosphokinase activity should correlate with development of contractility. Therefore, additional tests were made on skeletal muscle at 20th and 23rd day of foetal development. These tests revealed that the gastrocnemius muscle of 20 day old rabbit foetus contained creatine, no phosphocreatine, no creatine phosphokinase and did not respond to direct electrical stimulation. At this foetal age when the gastrocnemius gave no evidence of response to electrical stimulation, the same stimulus to the front legs elicited a good quick twitch-response. At 23rd gestational day, the gastrocnemius contained creatine, small amounts of phosphocreatine, first evidence of creatine phosphokinase and responded to direct electrical stimulation with a

TABLE I. Creatine Phosphokinase Activity in Heart and Skeletal Muscle of Foetal Rabbits.

$\mu$M phosphorus transferred from ATP to creatine/g tissue dry wt/min.

| Foetal age, days | No. pooled samples | Gastrocnemius | Heart |
|---|---|---|---|
| 14 | 8 | 0† | 0 |
| 16 | 6 | 0 | 32 ± 10* |
| 20 | 9 | 0 | 186 ± 15 |
| 23 | 6 | 68 ± 8 | 209 ± 22 |
| 30 | 10 | 382 ± 22 | 486 ± 20 |
| Age following birth, days | | | |
| 30 | 8 | 1600 ± 72 | 750 ± 36 |
| 60 | 9 | 1690 ± 64 | 780 ± 44 |
| 90 | 9 | 1180 ± 56 | 710 ± 40 |

* $\sqrt{\frac{\Sigma d^2}{N(N-1)}}$.

† Minimum limit of detectable activity is approximately 8 $\mu$M phosphorus transferred/g tissue/min.

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† Medical Student Fellowship of Nat. Fn.

weak slow worm-like contraction followed by an extremely slow incomplete relaxation. At 30 days of gestation, representing tissue collected immediately following birth or from the mother on 30th gestational day, creatine phosphokinase activity of the heart was approximately 60% of the adult level, while enzymatic activity of skeletal muscle was approximately 30% of adult level.

During 30 days following birth, the creatine phosphokinase activity of skeletal muscle rose rapidly, remained high through 60 days and fell to the adult level at 90 days, after which time it remained constant for at least 9 months. Enzymatic activity of heart tissue reached the adult level at 30 days of age and remained stable at this level.

*Discussion.* While some attempt was made to correlate appearance of phosphocreatine with onset of muscle contraction(4), Herrmann and Cox(5) concluded that in chick embryonic muscle, phosphocreatine appeared at a time when contractility was well developed. However, Koschtojanz and Rjabinowskaja(6) found that phosphocreatine in skeletal muscle of rabbit foetuses was first demonstrable at approximately the time of beginning response to pinprick.

In our investigation, the abrupt appearance of the enzyme creatine phosphokinase in gastrocnemius correlates well with development of contractility as evidenced by response to electrical stimulation. However absence of the enzyme in the beating heart muscle of 14 day rabbit foetuses militates against interpreting this correlation as evidence that the enzyme is necessary for contractility. Dwinnell(7) gave time of apparance of first heart beats in rabbit embryos as 8½ days, a full week before appearance of the enzyme.

Three possibilities should be considered. 1. Phosphocreatine and creatine phosphokinase are necessary for contractility in skeletal muscle but not in heart. This does not seem reasonable, for in all studies involving contractile proteins, those from skeletal muscle function qualitatively in the same manner as proteins of heart(8). 2. Appearance of creatine phosphokinase and phosphocreatine are correlated with development of nervous innervation and control rather than contractility. This problem is presently under investigation. 3. Development of contractility in muscle is dependent only on creatine phosphokinase which is closely associated with actin(9). The presence of actin bound enzyme appears to be involved in the phosphate transfer which takes place during actin polymerization and for formation of the contractile F-actinmyosin(10). The possibility exists that the actin bound creatine phosphokinase may be undetected by the present procedure due to the small amount of foetal tissue available for analysis. The sarcoplasmic creatine phosphokinase(11), in larger amounts, is detectable by our methods, although it may function only indirectly in muscle activity. This problem is presently being investigated and an attempt is underway to develop a more sensitive method of creatine phosphokinase assay.

*Summary.* Quantitative analysis of creatine phosphokinase in rabbit foetal tissue revealed that the enzyme is first detected in the heart on 16th day of gestation and in the gastrocnemius muscle on 23rd gestational day. There appears to be reasonable correlation between appearance of creatine phosphokinase and development of contractility in skeletal muscle but such a correlation does not exist in heart muscle.

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# New Types of Coxsackie Virus, Group A. Cytopathogenicity in Tissue Culture. (25383)

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Because of increasing concern about the role of Coxsackie Group A viruses in infection and as an aid in classification of the enteric viruses now so readily isolated, type numbers have been assigned to representative strains of 5 new types and their activity in tissue culture is described. Identification as Coxsackie virus, Group A, rests on infectivity for suckling mice, together with characteristic histopathology and signs of disease. Type determinations were made in suckling mice with hamster and mouse immune sera(1). *Type 20, 20(a), 20(b).* Serum prepared with each of the 3 representative strains neutralizes the others but the heterologous titer is in each case only approximately one-fourth of the homologous. Further experience is necessary to indicate whether all new isolations will show a degree of strain specificity or whether they will tend to be more like one or other of the representative strains. The representative strain for Type 20, No. 55166, was isolated in 1955 in this laboratory on human embryonic cell cultures and reisolated on HeLa cells by Dr. Sonja Buckley from a pool of fecal specimens from patients with infectious hepatitis. The Type 20(a) representative strain, No. 5536, originally isolated in 1951 in the laboratory of Dr. John Fox on human skin-muscle tissue culture from stool specimen of a suspected poliomyelitis patient, was received by us in 1955 from Drs. Edwin D. Kilbourne and Martin Goldfield of Tulane Univ. School of Medicine. Type 20(b) No. 5721 was isolated in 1951 in the laboratory of Dr. J. H. S. Gear, South Africa, from feces of a child living in Germiston Native Location. The representative strain of Type 21, No. 55161, was received in 1955 as $TC_4$ $SM_7$ from Dr. Edwin Lennette, Berkeley, Calif., with a history of isolation in human fetal tissue from stool material. This strain has occasionally induced paralysis in 10-12 g mice(2). Type 22 representative strain, No. 5630, was isolated in this laboratory directly in suckling mice from a stool specimen. Type 23 is the designation given to the virus at present generally known as ECHO 9. The first strain studied by us, No. 55222, was received from Dr. Italo Archetti, Rome, Italy, in Dec. 1955(3). Epidemic strains of this type do not usually infect suckling mice directly from the fecal specimen; tissue cultures do so in many cases but not in all(4,5,6). The virus chosen as the representative strain, No. 5844, however, was isolated in this laboratory directly in suckling mice from a stool specimen. The representative strain of Type 24, No. 5720, was isolated in 1952 from feces of a Germiston native baby in the laboratory of Dr. J. H. S. Gear, South Africa.

*Methods. Suckling mice.* Infectivity, type determination and cross reactivity tests were made in suckling mice. Typing sera were prepared in hamsters or large mice, using as antigen muscle suspension of suckling animals of the same species. Type 20 No. 55166, when first tested had little pathogenicity for suckling hamsters and a mouse serum was prepared with this strain. For the other strains, antisera were readily prepared in hamsters. *Tissue culture.* Human uterine tissue in plasma clot and trypsinized monkey kidney tissue were prepared as previously described (7). Medium 199 was used as maintenance medium for both types of culture. Two lines of HeLa cells were used, one received originally from Tuskegee Inst., the other from Dr. William Scherer. Both had been maintained in this laboratory for some time. For growth, 10% of human serum was added to a nutrient fluid prepared by adding 0.1% glucose, 0.1% yeast extract and 0.25% tryptose to Hanks' solution. This was replaced at time of virus inoculation by the yeast extract medium of Syverton and McLaren(8) containing 10% serum of which 2 parts were monkey

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* Deceased June 29, 1959.

TABLE I. Cytopathogenicity of Coxsackie Virus.

| | Tissue cultures | | | | |
|---|---|---|---|---|---|
| Type | Monkey kidney | Human uterine | HeLa | Human amnion | Trypsinized chick tissue |
| 20 | — | — | +* | — | — |
| 20(a) | — | — | +* | | — |
| 20(b) | — | — | +* | | — |
| 21 | — | + | + | + | — |
| 22 | — | — | — | — | — |
| 23 | +† | — | — | +† | |
| 24 | — | — | — | | — |

The inoculum for all cultures was a suspension of infected suckling mouse tissue.

* Types 20, 20(a), and 20(b) are cytopathogenic for both cell lines of HeLa used, but were more active on the line originally received from Tuskegee Inst.

† Type 23 is markedly cytopathogenic for monkey kidney, less so for human amnion.

serum and one part calf serum. Human amnion cell cultures prepared by trypsinization were grown in Bodian's medium(9) modified by omission of liquid bovine albumin and with addition of 20% human serum. For maintenance, the modified Bodian medium plus 2% calf serum was used. Chick embryo tissue cultures were prepared by trypsinizing 7 or 8 day embryos. Medium 199 plus 2% horse serum was used for growth; for maintenance the serum was omitted. Sterile sodium bicarbonate was used to adjust the reaction. Penicillin and streptomycin were added at time of use. The cytopathogenicity of virus strains was studied on various tissues but with the exception of Type 20 strains on HeLa cells, no attempt was made to adapt them.

*Results.* All strains induced flaccid paralysis in newborn mice with marked myositis but no significant lesions in the central nervous system. They caused no apparent disease in 10-12 g mice, with the exception of Types 21 and 24 which occasionally caused paralysis. On subsequent passages no paralysis was induced. Type 23 virus was not neutralized by Coxsackie A-9 hamster serum in neutralization tests in mice. Tyrrell and coworkers(10) noted that in complement-fixation tests the 2 types, while distinct, have some antigenic relationship.

The cytopathogenicity of the strains for various tissues is shown in Table I.

*Summary.* Five additional types of Coxsackie virus Group A have been assigned numbers, Types 20-24. Strains of all types with the exception of Type 23 are isolated readily in suckling mice. Type 23, which is the infectious agent presently known as ECHO 9, may be more easily isolated from the fecal specimen when tissue cultures are used for isolation. Types 20, 21 and 23 were cytopathogenic for one or more types of tissue in cultures; Types 22 and 24 did not induce damage on any of the cell types used.

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# Normal Growth of Mammary Gland in Pregnant and Lactating Mice.* (25384)

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Students of mammary gland growth in the past have been able to distinguish only lobule-alveolar development by morphological means (1,2). By measuring the amount of desoxyribonucleic acid (DNA)/gland(3,4,5), quantitative estimations of cell multiplication throughout pregnancy in the mouse(6) and further proliferation during lactation in the rat(7) have been observed. Our study presents a more detailed study of mammary gland development during pregnancy and lactation in albino mice. DNA values thus obtained can be used as a standard of reference in future studies concerning experimental growth of gland.

*Materials and methods.* Virgin, primipara, pregnant and lactating Webster-Swiss mice, fed a standard laboratory feed, were maintained at 78 ± 1°F. Ovariectomized lactating mice were operated on day 2 of lactation and sacrificed on day 10. Mice weighing 28-30 g were bred and pregnancy dated from morning vaginal plug. Animals mated but lacking fetuses were classified as pseudopregnant. Lactating mice were housed in individual cages with reduction of young to 5 shortly after parturition. Animals were sacrificed, skinned and 7 of the mammary glands of each mouse taken for DNA determination at time indicated (Table I). Remaining 3 glands were used for whole-mount observations. Glands were frozen immediately to inhibit enzyme (DNA-ase) activity(8). Tissues were thawed in 95% ethanol(4) and fat extracted from 5-8 hours, depending on adiposity of gland, placed in ethyl ether 4 hours, dried and ground to a fine powder in a Wiley mill. Standard sample (30 mg) of gland was extracted twice with 5 ml of hot trichloracetic acid to remove DNA which was then determined by the method of Webb and Levy(9).

*Results.* Highly significant differences (P .001) exist between the virgin total DNA level and that of 12 and 18 days pregnancy. Between 12- and 18-day pregnant mice a highly significant difference (P .001) was observed also.

There is a slight but non-significant increase in DNA of 6-day pregnant as compared to pseudopregnant mice. At 12 days pseudopregnancy, no further increase in DNA was observed, whereas DNA of 12-day pregnant mice increased 51.9%. Similar total DNA levels were noted between present results and earlier data obtained with virgin, pseudopregnant and pregnant mice(6). These comparisons indicate repeatability of mean DNA under standard laboratory conditions and the same strain. Total DNA increased gradually after parturition to maximum level of 10.211 ± .426 at 14 days lactation. DNA of days 5, 10, and 14 of lactation were statistically highly significant when compared to 18th day pregnancy. Decline in DNA on day 21 may result from partial glandular involution. In the lactating rat there was no significant decline in DNA from days 14 to 21 (7).

Lactating ovariectomized mice exhibited cellular proliferation comparable to normal lactators at 10 days.

*Discussion.* Past studies using morphological methods have been interpreted as indicating complete lobule-alveolar development by the first ½-⅔ of pregnancy. These data indicate, however, that lobule-alveolar growth continues throughout pregnancy and well into lactation. Of the total cellular increase, 30.6% occurred during the first 12 days of pregnancy, a further 47.5% was observed during second half of pregnancy and a final 21.9% increase noted by day 14 of lactation.

It had previously been assumed that growth during first ½ of pregnancy and pseudopregnancy was similar. These data show that growth is complete at 6 days in pseudo-

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TABLE I. Normal Growth of Mammary Gland in Pregnant and Lactating Mice.

| Reproductive state | Stage of pregnancy or lactation | No. of mice | DFFT,† mean (mg) | DNA ($\mu$g) mg of DFFT, mean ± S.E. | Total DNA (mg), mean ± S.E. | Final body wt, mean (g) | Total DNA (mg)/10 g final body wt, mean ± S.E. |
|---|---|---|---|---|---|---|---|
| Virgin | | 16 | 40.6 | 44.1 ± 2.1 | 1.757 ± .022 | 29.6 | .590 ± .002 |
| " * | | 7 | 44.0 | 38.8 ± 3.5 | 1.704 ± .180 | 30.8 | .544 ± .051 |
| Nullipara | 6 days after sterile coitus* | 15 | 52.5 | 42.9 ± .5 | 2.269 ± .126 | 31.6§ | .715‖ ± .038 |
| | 12 days after sterile coitus | 13 | 51.9 | 41.8 ± 1.2 | 2.147 ± .116 | 32.5 | .661 ± .047 |
| | *Idem** | 5 | 54.8 | 39.8 ± 1.4 | 2.202 ± .159 | 32.8§ | .778‖ ± .082 |
| Primipara | 6 days of pregnancy* | 10 | 63.2 | 46.3 ± 2.0 | 2.853 ± .153 | 31.4§ | .904‖ ± .049 |
| | 12 " " " | 14 | 95.3 | 46.9 ± 1.9 | 4.344 ± .261 | 36.9‡ | 1.171 ± .047 |
| | 12 " " " * | 10 | 80.1 | 51.4 ± 2.8 | 4.118 ± .319 | 32.8§ | 1.253‖ ± .091 |
| | 18 " " " | 15 | 125.6 | 57.0 ± 1.7 | 7.007 ± .281 | 38.5‡ | 1.832 ± .021 |
| | 18 " " " * | 10 | 121.8 | 49.4 ± 1.6 | 5.965 ± .353 | 32.0§ | 1.858 ± .070 |
| Primipara lactating | Immed. postpartum | 20 | 202.2 | 43.2 ± 1.7 | 8.358 ± .308 | 35.5 | 2.355 ± .080 |
| | 5 days lactating | 14 | 247.8 | 42.1 ± 3.0 | 9.758 ± .396 | 35.9 | 2.719 ± .104 |
| | 10 " " | 15 | 317.0 | 32.1 ± 1.1 | 9.988 ± .494 | 39.1 | 2.559 ± .105 |
| | 10 " " ¶ | 10 | 269.7 | 34.5 ± 1.4 | 9.135 ± .627 | 36.7 | 2.489 ± .045 |
| | 14 " " | 11 | 336.9 | 31.3 ± 1.8 | 10.211 ± .426 | 37.5 | 2.756 ± .114 |
| | 21 " " | 16 | 245.0 | 33.4 ± 1.7 | 7.742 ± .516 | 37.9 | 2.032 ± .113 |

* Data obtained from Wada and Turner, *J. Dairy Sci.*, 1959, v42, 1198.
† DFFT = Dry, fat-free tissue. ‡ Body wt corrected for wt of fetus. § Initial body wt.
‖ Total DNA (mg)/10 g initial body wt. ¶ Ovariectomized, day 2 of lactation.

pregnant mice, whereas in pregnancy the development of mammary gland continues. It is believed, therefore, that something associated with pregnancy is contributing to the stimulation of growth of gland not present in pseudopregnancy.

It was thought that corpora lutea of lactation contributed to additional lactational growth. However, the ovariectomized lactating animals sacrificed at 10 days exhibited DNA levels comparable to normal lactators. Therefore, growth of mammary gland during lactation is not dependent on the corpora lutea of lactation.

Some variability may be eliminated due to variation in body weight by using total DNA/body weight. In present experiment final body weights were quite similar, whereas for purposes of comparison with mice of varying body weight it is suggested total DNA be corrected for body weight.

*Summary.* 1) Using DNA as index of mammary gland growth, a study of changes from virgin throughout pregnancy and lactation in Webster-Swiss mice was conducted. Previous morphological studies were interpreted as indicating complete lobule-alveolar development by the first ½-⅔ of pregnancy. Our data show, however, only 30.6% of total growth during first 12 days of pregnancy, 45.7% in latter half of pregnancy and 21.9% further proliferation from parturition to day 14 of lactation. 2) Growth of the gland continues throughout pregnancy, but in pseudopregnancy it is completed by day 6. 3) DNA level of ovariectomized lactating mice is similar to normal lactators, indicating corpora lutea of lactation does not contribute to additional growth of mammary gland.

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# Thermal Behavior of the Rat Before and After Feeding. (25385)

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In a previous study(1) the effects of food deprivation on thermal behavior of the rat were investigated. Rats exposed to low ambient temperatures and trained to depress a response bar to obtain heat did so for longer periods of time, deprived than when fed *ad lib*. This increased use of external heat by the deprived rat was interpreted as a reflection of decreased energy supply. The present work is an extension of above study, investigating the effects of time of feeding in relation to testing time in 2 groups of rats maintained on a deprivation schedule.

*Method*. The animals were 12 male Sprague-Dawley rats with mean weight of 290 ± 6 g at beginning of study. The experimental apparatus has been described(1). Essentially it was a rectangular box of galvanized steel placed in conventional refrigerator with ambient temperature at –3 ± 1°C at beginning of experimental session. Heat was supplied by two 250 watt infrared lamps placed 4½″ from a response bar. Depression of the bar activated the heat lamps and a cumulative timer and response counter. The lamps remained on as long as the bar was depressed. Two response measures were recorded, total time that heat lamps were on and number of bar presses. Preliminary training consisted of placing each rat in the experimental apparatus for 1 hour a day. For the first 13 days all rats were placed on a restricted diet of approximately 10 g of chow pellets a day. The following 13 days all rats were allowed to feed *ad lib*. on powdered chow. For the next 16 days they were fed on powdered chow *ad lib*. but only for 2 hours following the one hour session in the cold. For the remaining 11 days of preliminary training all animals were fed during the 2 hours preceding, instead of after, the cold session. Then 2 groups were formed by counterbalanced order, on the basis of mean heat-on time of each rat over the last 7 days of preliminary training. Group A was fed 2 hours beginning 15 minutes after the one hour cold session. Group B continued as before, fed 2 hours just before the cold test. The groups were tested under these conditions for 10 consecutive days, then experimental conditions were reversed, with Group A fed before testing and Group B after, and continued for 10 additional days. Purina Lab Chow was used throughout.

*Results*. Fig. 1 summarizes results of the last 7 days of preliminary training and the experiment proper. During preliminary training phase, with all rats fed before testing, there were no significant differences between groups for mean heat-on times. On the first day of experiment the means of both groups increased, but the fed group less than the nonfed. (The reason for this increase in the fed group is not clear. It seems to emphasize the need for testing 2 groups because of the large intra group fluctuations of the daily means. However, whatever variables influenced the daily means, apparently had similar effects on both groups since the shape of the 2 curves is similar.) All 10 Group A means were above the Group B means, and on reversal of experimental conditions the opposite occurred. Assuming, on the basis of the training data, that the means are likely to be above or below each other in a random manner, the probability of all 10 means in one session falling as they did is $0.5^{10}$. The several instances of overlap of standard errors are attributed to the within group variability. This is not considered damaging since all animals when taken as their own controls showed a rise or fall in heat-on time when experimental conditions were changed.

It is apparent that the bar press rates did not discriminate between the 2 groups. The several days of high rates by the A group, during the last 10 days, are probably a reflection of their higher rates during the training period. The consistent difference between weight curves over the entire experiment does

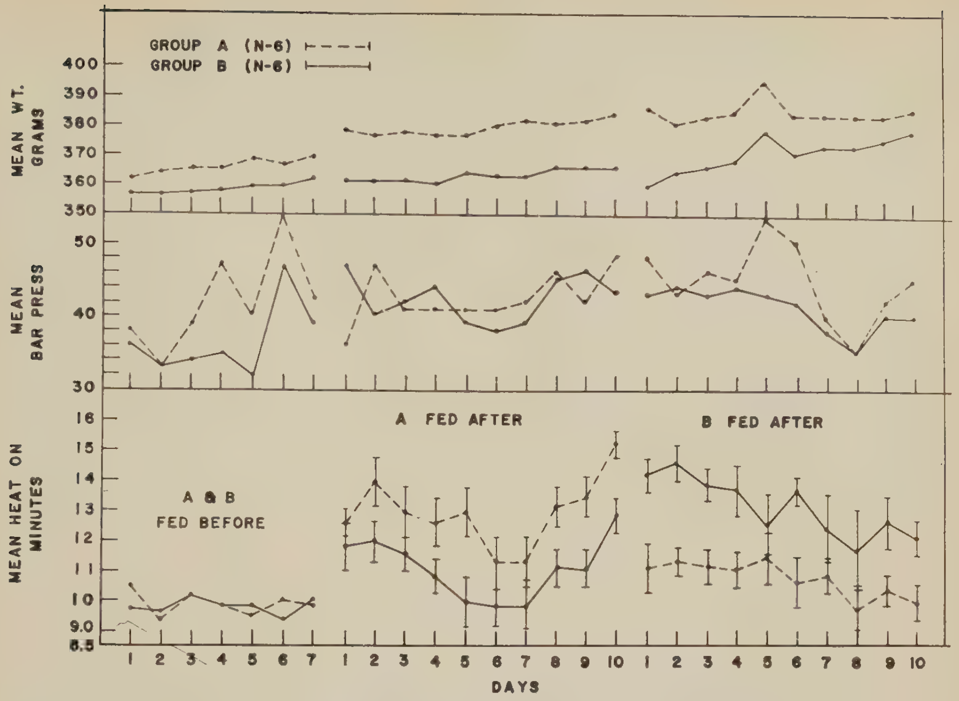

FIG. 1. Mean wt, bar press, and heat-on time as a function of experimental conditions. S.E. of means have been plotted for heat-on times.

not indicate a greater degree of deprivation for the B group since this group differed at beginning of the study. Weight and bar press rate were not controlled because heat-on time was the control variable.

There is little doubt that with 2 groups of rats kept on deprivation schedules, the group tested just after feeding generates lower heat-on time than those fed just before the test. One interpretation of these data would account for this difference by relating specific dynamic action (SDA) of food as the significant variable. That is, the increased heat production after feeding reduces the need for external heat and results in lower heat-on times. In studying the effects of SDA on heat production in the human, Glickman, *et al.*(2) report that accumulation of calories above fasting level postprandium, increases for 1.5 to 2.5 hours. This effect is highest after a meal relatively high in protein when it reaches 33 Kcal./hr above basal. However, these authors conclude that SDA has an inappreciable effect in protecting man in a cold environment. In studies reviewed by Burton and Edholm (3), high carbohydrate and high fat diets consistently were more effective than a high protein diet in maintenance of thermal balance and in performance of psycho-motor tests in men exposed to cold stress. Apparently the heat stress associated with SDA is dissipated in man through an increased cutaneous circulation. This would lead to increased rate of heat loss which would be detrimental with low ambient temperatures. Until this variable is investigated by manipulating the relative fat, carbohydrate, and protein content of the diet (the present diet was approximately 23% protein and 5% fat by volume) the effect of SDA on thermal behavior of the rat is open to speculation.

Another explanation of our data is suggested by Mitchell, *et al.*(4) who investigated the effects of diet on tolerance of man to cold. Their work shows high fat meals to be superior over high carbohydrate meals in protecting against cold only when the meals are taken at 2 hour intervals. (In this case the SDA of diets was assumed to be approximately the same.) They suggested that the reason for this difference may be that fat is temporarily deposited in subdermal tissues thereby reducing thermal conductivity. They base this reasoning on work by Schoenheimer and Rittenberg(5) which showed that in the mouse the bulk of dietary fat is conveyed to fat depots before utilization. As a consequence, the superiority of fat over carbohydrate and protein in protecting against cold relates more to its effects on reducing heat loss than to increased heat production. Resolution of these 2 explanations utilizing heat-on time data awaits further study, particularly with high protein (high SDA) diets. Sufficient at present is the demonstration that regarding the total organism, utilizing a learned response, there is definite evidence that regulation of environmental temperature is a function of time of feeding.

*Summary.* Rats exposed to low ambient temperatures were trained to depress a response bar to obtain heat. Rats tested for heat reinforcement before feeding pressed for more heat than those tested after feeding.

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## Carcinogenic Effect of Hens' Eggs as Part of the Diet in Mice. (25386)

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It has been shown that eggs are carcinogenic in chickens(1) and mice(2) and that their effect is not limited to a single organ or system. It appeared, however, that in "A" strain mice the systems most frequently affected by diet supplemented with eggs were respiratory and lymphoid. Out of 12 mice with malignancies, 10 had lung adenocarcinoma. Lymphoid organs (lymph nodes and spleen) were hypertrophied in majority of experimental mice. Three of them died of leukemia. The question is whether respiratory and lymphoid tissues are most susceptible systems to carcinogenesis in "A" strain or whether they are specifically affected by eggs in any strain of mice. To solve this problem more experiments of the same type were carried out upon "A" strain as well as other strains of mice. We report 3 series of experiments, 2 of which were carried out upon "A" strain and one upon "T.M." strain of mice. The latter is an inbred strain of mice developed in the School of Tropical Medicine (T.M.), from a pair of Swiss mice. During 10 years, according to Dr. C. Casas, no mice developed malignancies. It is considered a cancer resistant strain. This was the main reason for using it in present experiments. The series are referred to as second, third and fourth. The results of the first series have been described(2).

*Methods.* The second series consisted of 16 experimental mice (8 males and 8 females) and 16 controls (8 males and 8 females). The third series consisted of 16 experimental, 16 controls and 16 mice maintained on a Rockland rat diet supplemented with cottage cheese fed *ad lib.,* to find out whether a high protein diet had any effect upon carcinogenesis. Each of these 3 groups consisted of 8 males and 8 females. In above 2 series "A" strain mice were used. The fourth series was carried out upon "T.M." strain of mice, 16 experimental mice of 9 males and 7 females and 16 controls of 8 males and 8 females. In all 3 series controls were littermates, and males and females caged separately. Experimental mice were maintained on Rockland rat diet supplemented with hard boiled eggs at age of 4 weeks, immediately after weaning. Mice of second series received one egg daily during first month, 2 to 3 eggs daily thereafter. Mice

TABLE I. Time of Appearance, Location and Identity of Malignancies in Experimental Mice of Series 2.

| Sex | Age in mo | Mo on diet | Primary tumors | Location | Identity of tumor |
|---|---|---|---|---|---|
| ♂ | 10 | 9 | 3 | lungs | adenocarcinoma |
| | | | | liver | hepatoma |
| | | | | lymphoid | leukemia |
| ♂ | 11 | 10 | 1 | liver | hepatoma |
| ♂ | 12 | 11 | 1 | ” | ” |
| ♀ | 13 | 12 | 1 | lymphoid | leukemia |
| ♀ | 13 | 12 | 2 | lungs | adenocarcinoma |
| | | | | intestine | ” |
| ♂ | 17 | 16 | 1 | liver | hepatoma |
| ♀ | 18 | 17 | 2 | lungs | adenocarcinoma |
| | | | | lymphoid | leukemia |
| ♂ | 19 | 18 | 1 | ” | ” |
| ♂ | 20 | 19 | 1 | lungs | adenocarcinoma |
| ♂ | 21 | 20 | | ” | ” |
| | | | | liver | hepatoma |
| ♀ | 23 | 22 | 1 | lymphoid | leukemia |

of third and fourth series received only one egg daily throughout experiment. As mentioned previously(2), mice eat preferably the yolk, and very little of the white. Controls were maintained on Rockland rat diet only, no eggs.

All experimental and control animals were autopsied and the organs affected fixed, sectioned, stained with hematoxylin and eosin and studied microscopically. In each series, the experiment terminated when last mouse on diet supplemented with eggs, died. All remaining controls were then killed and autopsied. When the last experimental mouse of second series died, 10 controls were still alive. When the last experimental mouse of third series died, 14 controls and 9 mice on diet supplemented with pressed cottage cheese were alive. Finally, when the last experimental mouse of fourth series died, 9 controls were alive and apparently in good health.

*Results.* Out of 16 controls of second series only 2 mice developed cancer. Both died at age of 21 months, one, male, of an adenocarcinoma of the lungs and the other, a female, of an ascites tumor. Out of 16 mice on diet supplemented with eggs 11 mice developed malignancies at the age of 10 to 23 months (Table I). Out of 16 controls of third series, 3 mice developed malignancies. They were all killed at the age of 19 months. One male had a hepatoma, another male and one female had adenocarcinoma of the lungs. Out of 16 mice on the diet supplemented with pressed cottage cheese, 3 developed malignancies. One male, which died at age of 14 months, had a carcinoma of the salivary glands. The 2 other mice, both females, were killed at age of 19 months; both had malignant hepatomas. Out of 16 experimental animals of the third series 14 developed malignancies (Table II). Four of these mice died before age of 18 months, 8 at 18 months and 2 at 19 months. Out of 16 controls of the fourth series, only one female developed an adenocarcinoma of the lungs. It was killed at age of 24 months. Out of 16 experimental mice 15 developed malignancies, and in 10 of them more than one primary tumor was found. In this series (Table III) only 2 mice

TABLE II. Time of Appearance, Location and Identity of Malignancies in Experimental Mice of Series 3.

| Sex | Age in mo | Mo on diet | Primary tumors | Location | Identity of tumor |
|---|---|---|---|---|---|
| ♂ | 11 | 10 | 1 | lungs | adenocarcinoma |
| ♀ | 14 | 13 | 2 | ” | ” |
| | | | | lymphoid | leukemia |
| ♀ | 15 | 14 | 1 | lungs | adenocarcinoma |
| ♂ | 16 | 15 | 1 | ” | ” |
| ♀ | 18 | 17 | 1 | oviduct | fibrosarcoma |
| ♀ | ” | ” | 2 | lungs | adenocarcinoma |
| | | | | intestine | lymphosarcoma |
| ♀ | ” | ” | 1 | ” | ” |
| ♀ | ” | ” | 1 | ” | ” |
| ♀ | ” | ” | 1 | lungs | adenocarcinoma |
| ♂ | ” | ” | 2 | ” | ” |
| | | | | intestine | lymphosarcoma |
| ♂ | ” | ” | 2 | lungs | adenocarcinoma |
| | | | | lymphoid | leukemia |
| ♂ | ” | ” | 3 | lungs | adenocarcinoma |
| | | | | liver | hepatoma |
| | | | | lymphoid | leukemia |
| ♂ | 19 | 18 | 1 | lungs | adenocarcinoma |
| ♀ | ” | ” | 1 | ” | ” |

TABLE III. Time of Appearance, Location and Identity of Malignancies in Experimental Mice of Series 4.

| Sex | Age in mo | Mo on diet | Primary tumors | Location | Identity of tumor |
|---|---|---|---|---|---|
| ♂ | 16 | 15 | 1 | lungs | adenocarcinoma |
| ♀ | 17 | 16 | 1 | ” | ” |
| ♂ | 20 | 19 | 1 | ” | ” |
| ♀ | ” | ” | 2 | ” | ” |
| | | | | ” | peribronchial L-sarcoma |
| ♂ | 21 | 20 | 2 | ” | adenocarcinoma |
| | | | | peritoneum | lymphosarcoma |
| ♂ | ” | ” | 1 | intestine | ” |
| | | | | lungs | adenocarcinoma |
| | | | | skin | squamous cell carcinoma |
| ♂ | 22 | 21 | 1 | lungs | adenocarcinoma |
| ♀ | ” | ” | 4 | ” | ” |
| | | | | ” | peribronchial L-sarcoma |
| | | | | intestine | lymphosarcoma |
| | | | | skin | squamous cell carcinoma |
| ♂ | ” | ” | 1 | peritoneum | lymphosarcoma |
| ♂ | ” | ” | 3 | lungs | adenocarcinoma |
| | | | | intestine | lymphosarcoma |
| | | | | skin | squamous cell carcinoma |
| ♀ | 23 | 22 | 3 | lungs | adenocarcinoma |
| | | | | intestine | lymphosarcoma |
| | | | | liver | anaplastic carcinoma |
| ♀ | 24 | 23 | 2 | lungs | adenocarcinoma |
| | | | | mammary gland | ” |
| ♀ | ” | ” | 2 | lungs | ” |
| | | | | ” | peribronchial L-sarcoma |
| ♀ | ” | ” | 3 | ” | adenocarcinoma |
| | | | | ” | peribronchial L-sarcoma |
| | | | | intestine | lymphosarcoma |
| ♂ | ” | ” | 2 | lungs | adenocarcinoma |
| | | | | ” | peribronchial L-sarcoma |

died before age of 18 months; all others died later.

From these results it appears clear that the organs most frequently affected by diet supplemented with eggs are lungs and those of the lymphoid system. Among experimental animals of the 3 series, 31 developed adenocarcinoma of lungs. Leukemia or lymphosarcoma was found in 22 mice. In many animals of fourth series, in addition to adenocarcinoma, a peribronchial lymphosarcoma invaded the lungs.

It is very difficult to distinguish between leukemia and lymphosarcoma. Only those with a very large number of white cells in the blood vessels were diagnosed as leukemia. In some mice with leukemia, a white cell count of 60,000 to 70,000 was found shortly before death. Animals which died of lymphosarcoma of the lungs did not reveal an increase in white blood count a short time before death, when they already presented symptoms of their disease, like dyspnea and cachexia. In mice with leukemia and in those with lymphosarcoma, spleen and lymph nodes were considerably enlarged, while the thymus remained unchanged.

It should be added that in almost all experimental females the ovaries were atrophic, while testes in males were very active. In 2 experimental mice nodular hypertrophy of the adrenals was found. The liver, as observed previously(2), appeared abnormal in all experimental mice.

*Discussion.* The nature of the carcinogenic substance and its localization in the 2 components of the egg, yolk or white, are still unknown. According to Arscott(3), Arscott, Weswig and Schubert(4), Menge, Lillie and Denton(5) a growth substance, extractable with ethanol is present in the yolk of the egg. Hradek(6) found that this substance has the same characteristics as a substance extracted from tumors. From preliminary results of experiments, now being carried out, it appears that the carcinogenic substance in eggs is not limited to yolk only, but is also present in the white. The high protein of the diet does not seem to have any effect, since mice on the diet supplemented with cheese do not develop more malignancies than the controls.

*Summary.* It appears that there is no appreciable difference between "A" and "T.M." strains of mice in susceptibility to carcinogenic effect of eggs. In experimental mice of both strains the incidence of malignancies is very high, while among controls it is considerably lower in the "T.M." strain than in "A" strain. As in previous experiments(2), lungs and lymphoid tissue appear to be most susceptible to the carcinogenic effect of eggs. Malignancies of epidermal origin were not observed in "A" strain mice, while out of 16 experimental mice of "T.M." strain, 3 developed squamous cell carcinoma of the skin.

Some slides were examined by Dr. E. Koppisch, Univ. of Porto Rico School of Med., to whom I am greatly indebted.

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## Age of Donor and Host in Sex Histo-Incompatibility to Skin Isografts.* (25387)

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Sex histo-incompatibility to skin isografts among members of some inbred strains of mice, particularly in those of the A and $C_{57}$Bl strains seems to be a well established phenomenon(1-7). These observations demonstrate that while skin isografts performed from female to female, male to male and female to male are in most cases successful, those performed from male to female result in a homograft-like rejection. The rejection of the male skin isograft by the female appears to be immunological in nature as has been suggested by Eichwald *et al.*(8), Hauschka(9) and Snell(10) who have interpreted this phenomenon as due to the recipient's lack of the donor's isoantigen which originates from a gene located on the Y chromosome. This view has been recently substantiated by the demonstration that tolerance of male skin could be achieved in female mice of the A and $C_{57}$Bl strains, by injecting the females with isologous male spleen cells at birth(11-12) or during the post-neonatal period(13). Similar results were obtained by placing adult females with isologous males in parabiosis (13). However, recent observations made in our laboratories have indicated that while weanling female mice of the A strain not injected with isologous male spleen cells usually accept male skin isografts, older recipient females of the same strain similarly grafted with male skin reject this skin in most instances (14). This prompted us to further investigate this problem using female recipients of different ages which were grafted with skin from isologous donors of same approximate ages. Data are also presented from experiments designed to ascertain whether or not

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TABLE I. Skin Isografts from Male to Female in *Strain A* Mice of Different Ages.

| Groups | Male donor age (days)* | Female recipient (days)* | Total No. isografts | Successful grafts† No. | % |
|---|---|---|---|---|---|
| I | 42 ± 1 | 36 ± 1 | 20 | 15 | 75 |
| II | 46 ± 2 | 59 ± 1 | 20 | 8 | 40 |
| III | 216 ± 23 | 274 ± 16 | 17 | 0 | 0 |
| IV | 43 ± 2 | 228 ± 15 | 15 | 7 | 47 |
| V | 280 ± 20 | 37 ± 1 | 20 | 13 | 65 |

* Mean and stand. dev.

† Successful grafts were maintained during the entire period of observation, ranging from 5 mo to 1 yr following grafting.

mice of some other inbred strains raised in our laboratories, namely those of the Z,[‡] NH, Ce and Balb/C, exhibit the phenomenon of sex histo-incompatibility.

*Materials and methods.* In a first set of experiments, female mice of the A and $C_{57}$Bl (subline 1) strains were divided into different groups according to age at time of receiving the male skin isograft. There were set up 5 groups of A strain animals and the ages of both recipients and donors are listed in Table I. Two groups of $C_{57}$Bl (subline 1) females received skin grafts taken from isologous male donors of the same age (Table II). In a second set of experiments, male → female, female → male, male → male and female → female skin isografts were performed in young mice of the following inbred strains: Z($C_3$H), Ce, Balb/C and NH. Ages of both recipient and donor animals at time of skin grafts are listed in Table III. In all instances a full-thickness 2 x 2 cm abdominal skin graft taken from the donor was transplanted to the dorsal surface of the recipient according to procedures previously described by Martinez *et al.*(15). Interrupted silk sutures were used to obtain intimate contact of the skin transplant with the surrounding host tissue. The graft was turned 180° to facilitate judgment of its subsequent success or failure since the successful skin graft hair grows in the opposite direction to the hair growth of the host. Grafted mice were housed in individual plastic cages and observed for periods ranging between 5 to 12 months.

*Results. Skin isografts from male to female mice of A and $C_{57}$Bl (subline 1) strains at different ages.* The results obtained for A strain mice are listed in Table I. In Groups I, II and III in which ages of both donors and recipients were approximately the same, incidence of successful grafts was significantly higher when grafts were performed between young animals (Group I) than when performed in older animals (Groups II and III). On the other hand, when young females were grafted with skin taken from old donors (Group V) incidence of successful grafts was almost the same as compared to the group of young recipients and young donors (65% and 75% respectively).

However, contrary to expectation, in old recipient females grafted with skin from young males (Group IV) incidence of successful grafts was significantly higher than in the old to old group (47% and 0% respectively).

Results obtained in groups of male to female isografts in $C_{57}$Bl (subline 1) strain, are listed in Table II. The overall incidence of successful male to female skin grafts was approximately the same in young (19%) as it was in old animals (13%). However, as indicated in the Table, there was a clear dif-

TABLE II. Incidence of Male Skin Isograft Rejection in Young and Old Female Mice of the $C_{57}$Bl (Subline 1) Strain.

| Strain | Recipient ♀ and donor ♂ age, range (days) | 8 day | % | 13 day | % | 18 day | % | 23 day | % | 33 day | % |
|---|---|---|---|---|---|---|---|---|---|---|---|
| $C_{57}$Bl (subline 1) | 29–34 | 4/16* | 25 | 5/16 | 31 | 11/16 | 69 | 11/16 | 69 | 13/16 | 81 |
| ” | 180 | 21/24* | 87 | 21/24 | 87 | 21/24 | 87 | 21/24 | 87 | 21/24 | 87 |

* No. of female mice showing complete sloughing of male isograft / Total number of mice grafted.

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[‡] Z is used to simplify designation of mice of $C_3$H strain obtained in 1956 from Dr. J. J. Bittner's mouse colony.

TABLE III. Skin Isografts from Male to Female, Female to Male, Male to Male and Female to Female in Several Inbred Strains of Mice.*

| | ♂ to ♀ | | | | ♀ to ♂ | | | | ♂ to ♂ | | | | ♀ to ♀ | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Strain | No. takes /total No. grafted | % | Recipient Age† | Donor Age† | No. takes /total No. grafted | % | Recipient Age† | Donor Age† | No. takes /total No. grafted | % | Recipient Age† | Donor Age† | No. takes /total No. grafted | % | Recipient Age† | Donor Age† |
| $Z(C_3H)$ | 21/21 | 100 | 48 | 35 | 16/16 | 100 | 41 | 41 | 23/25 | 92 | 47 | 47 | 19/19 | 100 | 36 | 36 |
| Ce | 6/13 | 46 | 46 | 49 | 7/7 | " | 44 | 44 | 16/16 | 100 | 42 | 42 | 7/7 | " | 50 | 50 |
| Balb/C | 17/20 | 85 | 55 | 54 | 20/20 | " | 60 | 60 | 14/14 | " | 40 | 46 | 23/23 | " | 39 | 39 |
| NH | 6/20 | 30 | 43 | 45 | 8/9 | 89 | 40 | 40 | 21/21 | " | 46 | 46 | 11/12 | 92 | 51 | 51 |

* Successful grafts were maintained during the entire period of observation, ranging from 5 mo to 1 yr following grafting.

† Avg age in days.

ference between both groups in reference to the time at which complete sloughing of the male skin graft occurred. This was significantly longer in the group of young females as compared to the old group.

*Sex histo-incompatibility to male skin isografts in mice of the $Z(C_3H)$, Ce, Balb/C and NH.* These results are listed in Table III. All of the $Z(C_3H)$ females accepted the male skin isografts. Similar incidence of successful grafts were obtained in mice of this strain when female → male, male → male and female → female skin transplants were attempted. Of 13 female mice of the Ce strain, transplanted with skin taken from isologous males, only 6 or 46% accepted this skin. On the other hand, skin isotransplants performed from females → males, males → males and females → females were all successful.

In the group of Balb/C strain mice, 17 out of 20 females accepted skin grafts taken from isologous male donors. Here again skin transplants performed from female → male, male → male and female → female were all successful.

Finally in mice of the NH strain, the results were as follows: Of 20 female mice grafted with skin taken from isologous male donors only 6 or 30% accepted the graft. Nearly all attempts to graft skin from females → males, males → males and females → females of this strain were successful.

*Discussion. Skin isografts from male to female mice of A and $C_{57}Bl$ (subline 1) strains of different ages.* The results of these experiments are of interest from several points of view. First, they demonstrate that in mice of the A strain, success or failure of isologous skin grafts performed from males to females depends to a certain extent on age of both recipient and donor at time the skin graft is attempted.

Interpretation of these results is not yet clear. One possibility might be that young recipient females of this strain within certain age range limits might not be fully immunologically mature and therefore not capable of recognizing or rejecting the corresponding male tissue. This would also explain previous observations indicating the possibility of inducing immunological tolerance of male skin

TABLE IV. Skin Isografts from Old Males to Old Females of Several Inbred Strains.*

| Strain | No. takes/total No. grafted | Recipient Age range (days) | Recipient Avg age (days) | Donor Age range (days) | Donor Avg age (days) |
|---|---|---|---|---|---|
| Z ($C_3H$) | 9/9 | 151–332 | 185 | 274–365 | 335 |
| Ce | 2/6 | 365 | 365 | 365 | 365 |
| Balb/C | 12/14 | 158–310 | 196 | 169 | 169 |
| NH | 3/17 | 365 | 365 | 173–365 | 254 |

* Successful grafts were maintained during entire period of observation, ranging from 5 mo to 1 yr following grafting.

isografts in weanling and in 3 month old females by injecting them intravenously with isologous male spleen cells(13). Another possibility might be related to the weakness of the histocompatibility barrier between males and females of this strain. In this regard it would be feasible to assume that this difference is stronger in mice of the $C_{57}$Bl (subline 1) strain as compared to that in A strain animals, since only a definite prolongation of the rejection time was seen in young $C_{57}$Bl females grafted with skin taken from young males as compared to the groups in which old to old combination was used.

The fact that the skin grafted in the old to old combinations was thicker than that used in the young to young, and in some way may have caused establishment of an adequate circulation to be impaired or delayed, might account for the shorter rejection time in this group.

*Sex histo-incompatibility to male skin isografts in mice of Z($C_3H$), Ce, Balb/C and NH.* The results of this experiment clearly demonstrate that of the strains investigated only mice of the Z($C_3$H) and Balb/C strains do not show the so-called Eichwald and Silmser phenomenon of sex histo-incompatibility. In fact all attempts made to graft male skin on isologous females of the Z($C_3$H) strain were successful. Similarly, mice of the Balb/C strain did not show this phenomenon since 85% of the female recipients transplanted with isologous male donor skin accepted this graft. On the other hand, sex histo-incompatibility to male skin isografts was clearly seen in mice of the Ce and NH stocks. There are no previous reports in the literature concerning results using mice of the NH and Ce strains.

Our results obtained in Z($C_3$H) and Balb/C mice although in agreement with similar observations reported by Prehn and Main in these 2 stocks(2), are at variance with those reported by Sachs and Heller(4) who found that all $C_3$H as well as Balb/C mice rejected the male skin isograft. These discrepancies in apparently the same strains of mice might be explained as due to different genetic make up of our Z($C_3$H) and Balb/C mice as compared to those used by Sachs and Heller. Our stocks were separated in 1956 from those maintained by Dr. John J. Bittner and have been raised in our laboratories with rigorous inbreeding.

*Summary.* 1) Age, as a factor in immunological maturity of both donor and recipient, is presented as a possible controlling factor in acceptance or rejection of skin isotransplants in the inbred A strain of mice. 2) The age factor does not play a significant role in skin isotransplants of the inbred $C_{57}$Bl (subline 1) strain. 3) A sex histo-incompatibility exists in inbred mice of the NH and Ce strains.

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# Mechanism of Bovine Plasminogen Activation by Human Plasmin and Streptokinase. (25388)

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It has been known for some time that conversion of bovine plasminogen to plasmin can be effected by addition of streptokinase in presence of trace amounts of either human plasminogen or plasmin. This is in contrast to activation of human plasminogen, elicited by addition of streptokinase alone. In both systems, the formation of plasmin has been ascribed by some investigators(1,2,3) to conversion of a specific proactivator by streptokinase to a singular activator which then acts on plasminogen to produce plasmin. Other investigators(4,5,6), however, have expressed the possibility that the proactivator of bovine plasminogen, when activation mixtures of streptokinase and human plasminogen or plasmin are used, may be identical with either human plasminogen or plasmin. The following studies were performed to examine the mechanism of bovine plasminogen conversion by human plasmin and streptokinase under various experimental conditions, and to determine whether this conversion is brought about by a specific activator principle, formed by action of streptokinase on a separate distinct proactivator, or whether streptokinase may form a complex with human plasmin which can directly convert bovine plasminogen to plasmin.

*Materials and methods. Human plasmin.* This enzyme was obtained from human plasma Fraction III[†] by a method previously described(7). *Bovine plasminogen.* Twenty-four liters of freshly collected bovine blood were permitted to clot at room temperature; after 2 hours standing, the clots were dispersed and strained through gauze. Final serum clarification was obtained by centrifugation for 30 minutes at 2,000 rpm at 0°C. The serum obtained (approximately 8 liters) was cooled to 0°C and brought to 25% ammonium sulfate saturation by addition of pre-cooled saturated ammonium sulfate solution, stirred for 30 minutes, then left for 1 to 2 hours at 0°C. The precipitate obtained following centrifugation was discarded and the supernatant solution adjusted to 33% saturated ammonium sulfate by further addition of pre-cooled saturated ammonium sulfate solution at 0°C. After remaining overnight at 4°C, the precipitate was collected by centrifugation, dispersed in distilled water, and dialyzed against running tap water for 18 hours at 4°C. The precipitate formed on dialysis was collected by centrifugation, dispersed in 100 to 150 ml of 0.9% sodium chloride and then diluted 1:8 with distilled water at room temperature. To achieve maximum solubilization, pH was adjusted to 9.4 with 1 N sodium hydroxide, and the solution was then brought to pH 5.3 with 1% acetic acid. The precipitate that formed was allowed to settle overnight at 4°C. After centrifugation, the precipitate was dissolved in 1 liter of phosphate buffer pH 7.0, ionic strength 0.15. The solution was cooled to 0°C and brought to 25% saturated ammonium sulfate by addition of pre-cooled satu-

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[*] Died Sept. 30, 1959.

[†] Obtained through courtesy of Am. Nat. Red Cross, Washington, D.C., and kindly supplied us by Cutter Labs., Berkeley, Calif., as well as by E. R. Squibb and Sons, New Brunswick, N. J.

rated ammonium sulfate solution. After centrifugation, the supernatant solution was adjusted to 33% ammonium sulfate saturation. The precipitate obtained after centrifugation was dispersed in distilled water, dialyzed against running tap water and then against distilled water at 4°C; the water insoluble material was removed by centrifugation, dispersed in distilled water and lyophilized. In all experiments, a freshly prepared solution of 5 mg of bovine plasminogen in 1 ml of phosphate-sodium chloride buffer, pH 7.0, ionic strength 0.15, was utilized. *Streptokinase.*‡ Solutions of desired concentrations were made with 0.9% sodium chloride, pH 7.0. All stock solutions were stored at 4°C and kept no longer than one week. *Casein.* One per cent stock solutions of casein were prepared (7), either in saline-phosphate buffer (0.1 M phosphate, 0.9% saline), pH 7.4 or in 0.1 M phosphate buffer, pH 7.6. *Crystalline pancreatic trypsin inhibitor.*§ Stock solutions of 4, 6, 8, and 12 μg/ml were prepared in 0.1 M phosphate buffer, pH 7.0. *Standard test procedure.* On the basis of preliminary studies, the following standard procedure was devised. A 1 ml incubation mixture containing 0.05 mg of human plasmin and 250 units of streptokinase was obtained by addition of 0.5 ml of 0.1 mg/ml plasmin solution in 0.001 N hydrochloric acid to 0.5 ml of 500 units/ml streptokinase solution prepared in 0.9% saline adjusted to pH 7.0. The reaction at pH 6.6 proceeded at room temperature 5 to 10 minutes prior to addition to 1 ml of a 5 mg/ml bovine plasminogen solution freshly prepared in saline-phosphate buffer (pH 7.0, ionic strength 0.15). Following 10 minute activation at room temperature for the mixture of human plasmin, streptokinase and bovine plasminogen, 2 ml of a 1% casein solution in saline-phosphate buffer, pH 7.4, prewarmed to 35°C were added to the mixture, and proteolysis of casein proceeded at 35°C for 30 minutes prior to addition of 5 ml of 5% trichloroacetic acid. Blanks were prepared by immediate addition of trichloroacetic acid prior to addition of casein. Extent of proteolysis was determined by reading optical densities of the trichloroacetic acid filtrates relative to the blanks at 280 mμ. Proteolytic units were determined from standard calibration curves (7). Schematically, the details of activation, conversion, and proteolysis can be summarized as follows:

| | | | |
|---|---|---|---|
| (1) Activation mixture:<br>(1 ml; 5 to 10 min.) | Human plasmin<br>(0.05 mg)<br>(0.5 ml) | + | Streptokinase<br>(250 units)<br>(0.5 ml) |
| ↓ | | | |
| (2) Conversion mixture:<br>(2 ml; 10 min.) | Bovine plasminogen<br>(5 mg/ml)<br>(1 ml) | + | Activation mixture<br>(1 ml) |
| ↓ | | | |
| (3) Digestion mixture:<br>(4 ml; 30 min.) | 1% casein<br>(2 ml) | + | Conversion mixture<br>(2 ml) |

*Effect of acid pH on the plasminogen activation.* Four 80 ml stock solutions (0.9% sodium chloride, pH 6.6) were prepared, containing 0.05 mg human plasmin/ml and the following units of streptokinase/ml, 1) 31.25; 2) 62.5; 3) 125; and 4) 250. Ten ml aliquots were withdrawn from each stock solution and quadruplicate assays at each streptokinase level were performed for activator potency by addition to bovine plasminogen under standard test conditions. The remaining solutions were adjusted to pH 2.0 by addition of 4 N hydrochloric acid, were permitted to equilibrate for 30 minutes at room temperature, then readjusted to pH 6.6 by addition of 4 N sodium hydroxide. Ten ml aliquots were removed for activator assays. Streptokinase, contained in 0.1 ml of 0.9% saline, pH 7.0, was added to each stock solution in initial units of streptokinase employed for that series. Aliquots were removed for assay. Once again the pH was reduced, readjusted, and an aliquot removed for assay. The entire procedure was repeated 3 times, utilizing original stock solutions throughout. The protocol has been summarized at top of Fig. 1, in which results have been graphically recorded. *Influence of pancreatic trypsin inhibitor on formation of activator.* Stock solutions in the following volume relationships were prepared: 1 ml of human plasmin : 0.5 ml of crystalline pancreatic trypsin inhibitor solution : 0.5 ml streptokinase solution : 2 ml of bovine plasminogen solution (5 mg/ml). Final concentrations of reactants in the 4 ml stock solutions were as follows: 0.2 mg human plasmin; 2, 3, 4, or 6 μg of inhibitor 125, 250, or 500 units of streptokinase; and 10 mg of bovine plasminogen. Streptokinase, inhibitor, and plasminogen were prepared in 0.1 M phosphate buffer, pH 7.0, while plasmin solutions were made with 0.0025 N hydrochloric acid. In all series careful consideration was given to any volume changes so that all series would be comparable. Similarly, accurate control of equilibration times for each series was maintained. Briefly, this was as follows: 20 minutes equilibration for inhibitor addition; 10 minutes each for the reaction with streptokinase and with plasminogen. The inhibitor was added at various stages of the reaction system as follows: Series 1: Controls; no inhibitor added. Series 2: Inhibitor added to human plasmin prior to addition of streptokinase and then of plasminogen. Series 3: Inhibitor added after human plasmin and streptokinase were allowed to react, and preceding addition to plasminogen. Series 4: Inhibitor added after human plasmin plus streptokinase had reacted with plasminogen but prior to addition to casein. Series 5: An additional series was devised in which human plasmin plus inhibitor and streptokinase were allowed to react, then additional human plasmin was added prior to mixing with plasminogen. Casein proteolysis was carried out according to Kunitz's procedure. One ml aliquots of varying reaction mixtures were added to 1 ml of 1% casein solution prepared in 0.1 M phosphate buffer, pH 7.6, prewarmed to 35°C and incubated at 35°C for 20 minutes prior to addition of 3 ml of 5% trichloroacetic acid. Blanks were prepared by immediate addition of trichloroacetic acid prior to casein addition. Extent of proteolysis was determined by reading optical densities of the trichloroacetic acid filtrates relative to blanks at 280 mμ. Four inhibitor concentrations of 2, 3, 4, or 6 μg, were employed for each of the 3 different streptokinase concentrations. The reactants were assayed for intrinsic proteolytic activity, but since no significant contributions to proteolytic activity by individual reactants were evident, the results are not listed. Fig. 2 contains both the protocol and results of this experiment. The protocol for each series is listed on top and the demonstrable proteolytic activity, as expressed in O.D. units, is listed on side of chart.

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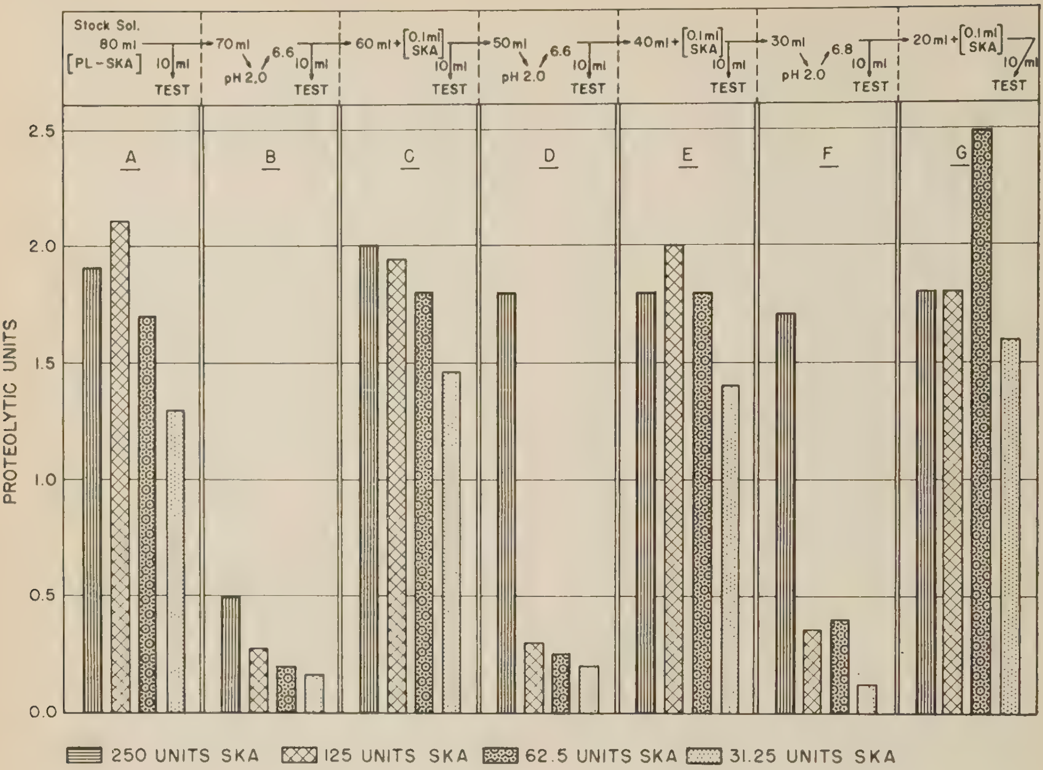
‡ Streptokinase-streptodornase (Varidase) was supplied through the generosity of Lederle Labs. Division, Amer. Cyanamid Co., Pearl River, N. Y.

§ Obtained from Worthington Biochemical Corp., Freehold, N. J.

*Results.* In the standard test procedure, a solution of human plasmin plus streptokinase following preliminary incubation was added to a solution of bovine plasminogen, the mixture allowed to react and then casein added. Three reactions proceed: Formation of an activator, conversion of bovine plasminogen by the activator, and proteolysis of casein by bovine plasmin formed. Addition of incubated solution of bovine plasminogen, containing human plasmin and streptokinase, to a casein substrate has been used to follow the activating effect of human plasmin plus streptokinase on bovine plasminogen. The amount of human plasmin employed was relatively small and produced no significant proteolysis when tested alone against casein. There was no conversion of bovine plasminogen to plasmin in either the presence of human plasmin or streptokinase alone; activation of bovine plasminogen was observed only in presence of both human plasmin and streptokinase.

Fig. 1 summarizes results of the effect of pH 2 on bovine plasminogen conversion activity. The values represent an average of duplicate determinations for at least 4 experiments. Generally, there was a corresponding increase in activator potency with increasing streptokinase concentration (column A). Following low pH treatment, there was a striking decrease in activator capacity (column B). Readdition of streptokinase returned activator levels to the pre-acid treatment values (compare column C with column A). With exception of 250 units streptokinase level, subsequent low pH equilibrations reduced activator potency again, while readdition of streptokinase returned the activator levels to original values (columns E and G). At a concentration of 250 units of streptokinase, the second and subsequent low pH treatments did not result in an apparent loss of activator potency (columns D and F). While there may have been destruction of ac-


Stock Sol. 80 ml [PL-SKA] 10 ml TEST
70 ml pH 2.0 6.6 10 ml TEST
60 ml + [0.1 ml SKA] 10 ml TEST
50 ml pH 2.0 6.6 10 ml TEST
40 ml + [0.1 ml SKA] 10 ml TEST
30 ml pH 2.0 6.8 10 ml TEST
20 ml + [0.1 ml SKA] 10 ml TEST
A
B
C
D
E
F
G
PROTEOLYTIC UNITS
0.0
0.5
1.0
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2.0
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250 UNITS SKA
125 UNITS SKA
62.5 UNITS SKA
31.25 UNITS SKA

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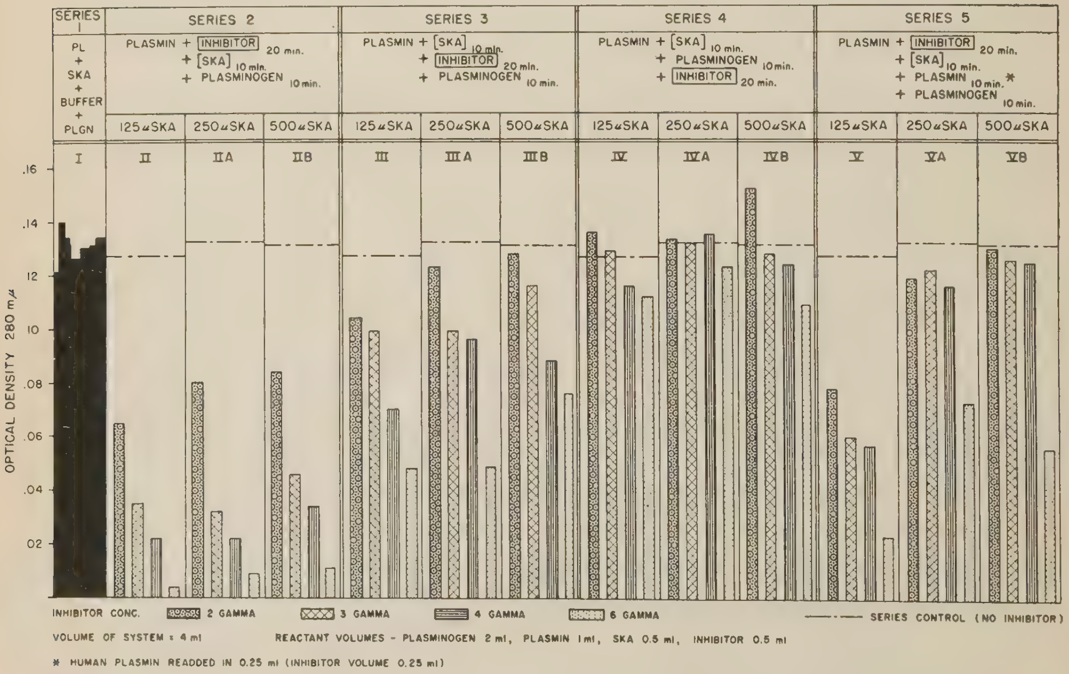

SERIES 1
PL + SKA + BUFFER + PLGN
SERIES 2
PLASMIN + INHIBITOR 20 min. + [SKA] 10 min. + PLASMINOGEN 10 min.
SERIES 3
PLASMIN + [SKA] 10 min. + INHIBITOR 20 min. + PLASMINOGEN 10 min.
SERIES 4
PLASMIN + [SKA] 10 min. + PLASMINOGEN 10 min. + INHIBITOR 20 min.
SERIES 5
PLASMIN + INHIBITOR 20 min. + [SKA] 10 min. + PLASMIN 10 min. * + PLASMINOGEN 10 min.
125 u SKA
250 u SKA
500 u SKA
I
II
IIA
IIB
III
IIIA
IIIB
IV
IVA
IVB
V
VA
VB
OPTICAL DENSITY 280 mμ
.02
.04
.06
.08
.10
.12
.14
.16
INHIBITOR CONC.
2 GAMMA
3 GAMMA
4 GAMMA
6 GAMMA
SERIES CONTROL (NO INHIBITOR)
VOLUME OF SYSTEM = 4 ml
REACTANT VOLUMES - PLASMINOGEN 2 ml, PLASMIN 1 ml, SKA 0.5 ml, INHIBITOR 0.5 ml
* HUMAN PLASMIN READDED IN 0.25 ml (INHIBITOR VOLUME 0.25 ml)

②

tivator, higher concentration of streptokinase in excess of non-reacted streptokinase could probably re-form activator after alkaline adjustment of the solution.

Plasmin as well as streptokinase were quite stable when incubated separately at low pH for 2 to 3 hours, since no decrease in activator activity was observed upon mixing after adjustment to pH 6.6 and addition to bovine plasminogen. The destruction of plasminogen activator activity following low pH treatment is in agreement with similar observations by others(5,8,9). However, the present results do not seem to support the concept that streptokinase interacts with a specific proactivator resulting in formation of a distinct singular plasminogen activator(1,2,3), since one would expect that repeated acid treatments would eventually lead to a decrease in activator activity, which was not observed.

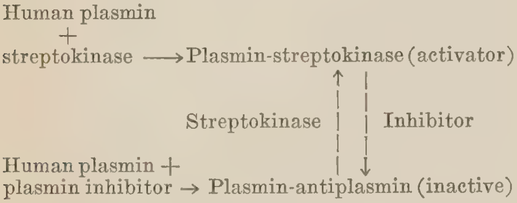
Fig. 2 summarizes results of 4 duplicate determinations, graphically illustrating the influence of a plasmin inhibitor on formation of plasminogen activator activity. Preliminary experiments showed that the pancreatic trypsin inhibitor had the capacity to inhibit proteolytic activity of human plasmin and that this inhibition for the amount of plasmin used was complete at higher concentrations of inhibitor employed. Column I indicates plasmin activity formed (as indicated by O.D. of the trichloroacetic acid soluble filtrates) when no inhibitor had been introduced at any stage in the test system. Columns II, II-A, and II-B demonstrate the marked decrease in activator potency when the inhibitor was added to plasmin prior to addition of streptokinase at the 3 levels employed. As the inhibitor concentration was increased, the decrease in activator formation became more evident. These results seem in agreement with the assumption that human plasmin may interact with streptokinase forming the bovine plasminogen activator. However, it was possible that the inhibitor may have interacted with the plasminogen activator rather than with plasmin. Experiments in which the inhibitor was added after interaction of human plasmin and streptokinase examine this aspect (columns III, III-A, and III-B). Although evidence of some decreased bovine plasminogen activation was manifested, this decrease in activator activity was definitely less than that observed when plasmin was first interacted with the inhibitor (columns II, II-A, and II-B). Once again, the decrease in activator activity was more marked as inhibitor concentration was increased. The decrease in activator activity under these experimental conditions may be due to an inhibition of the activator by the pancreatic inhibitor or to a replacement of some streptokinase in a plasmin-streptokinase complex. In the latter case, streptokinase apparently competes with the inhibitor in combining with human plasmin. If this were so, more pronounced activator activity might be expected as the ratio of streptokinase to inhibitor is increased. The data (columns III and III-B) seem to support this possibility. To determine that there was actually a decrease in activator activity and in resultant bovine plasminogen conversion in presence of inhibitor rather than any inhibition of plasmin formed from bovine plasminogen, the inhibitor was added after activation of bovine plasminogen. As can be seen from the data, illustrated in columns IV, IV-A, and IV-B, there was only a slight decrease in plasmin activity (more pronounced at 6 $\mu$g level of inhibitor) when compared with control series (column I). In the final experiments, human plasmin was readded after incubation of plasmin and inhibitor followed by streptokinase addition. At the level of 125 units of streptokinase (column V) there was more reactivation in the presence of 2 $\mu$g of inhibitor as compared with 3, 4, or 6 $\mu$g of inhibitor. At the levels of 250 (column V-A) or 500 (column V-B) units of streptokinase, with the exception of the 6 $\mu$g inhibitor concentration, reactivation was essentially complete. These findings would indicate that as long as there is no great excess of inhibitor, complete reactivation of the system is possible upon readdi-

FIG. 1. Effect of low pH on conversion of bovine plasminogen to plasmin by human plasmin and streptokinase.

FIG. 2. Effect of a plasmin inhibitor on conversion of bovine plasminogen to plasmin by human plasmin and streptokinase.

tion of human plasmin. Apparently streptokinase will compete with the inhibitor in combining with human plasmin.

Our results may suggest that human plasmin and streptokinase combine to form a complex which has the capacity to convert bovine plasminogen to plasmin. The following scheme may serve to illustrate the formation of activator and the influence of the plasmin inhibitor on this reaction:


Human plasmin
+
streptokinase ⟶ Plasmin-streptokinase (activator)
Streptokinase
Inhibitor
Human plasmin +
plasmin inhibitor → Plasmin-antiplasmin (inactive)

It is assumed that in the presence of plasmin inhibitor, streptokinase will compete with the inhibitor in combining with plasmin. The amounts of the plasmin-streptokinase or plasmin-antiplasmin complexes formed are dependent upon concentrations of streptokinase and of plasmin inhibitor.

*Discussion.* Our data do not seem to support the concept of a separate proactivator which under the influence of streptokinase is converted to a specific singular plasminogen activator(1,2,3). It may be pointed out that the postulated proactivator has not as yet been definitely demonstrated to be an entity, except by indirect inference. Our attempts to separate proactivator activity from human plasmin or plasminogen have thus far been unsuccessful. The data also do not substantiate the assumption that proactivator and human plasminogen are identical(6). Our results are in agreement with the postulation that human plasmin reacts with streptokinase perhaps proportionally, to form a plasmin-streptokinase complex which has the ability to convert bovine plasminogen to plasmin. The complex may contain streptokinase in a form so altered (possibly by preliminary reaction of plasmin on the streptokinase prior to complexing) that upon dissociation by acid, the bound streptokinase has become acid labile. This concept would be in agreement with the finding that activator activity can be destroyed by acid treatment and can be restored by subsequent addition of streptokinase after pH adjustment to 7.2.

Studies on the influence of a plasmin inhibitor, added to various stages involved in bovine plasmin formation, on final proteolytic activity indicate that apparently streptokinase can compete with the plasmin inhibitor in combining with human plasmin. Amounts of plasmin-streptokinase or plasmin-antiplasmin complexes formed seem to depend upon concentrations of streptokinase and plasmin inhibitor.

The formulation of a plasmin-streptokinase complex as the bovine plasminogen activator is in accord with the observation of Kline and Fishman(5) that there was no significant change in the ratio of proactivator to plasminogen (human) during purification of plasminogen as well as with the finding of Ablondi and Hagan(6) that there was no difference in heat stability between proactivator and human plasminogen. The proposed scheme of bovine plasminogen activation by human plasmin in the presence of streptokinase is in accord with similar schemes proposed by Kline and Fishman(5) as well as by Sherry and his associates(4). Meyers and Burdon(10) have shown that plasminogen in sera from a large number of different animal species could be activated with human plasminogen-streptokinase mixtures.

*Summary.* Equilibration of human plasmin-streptokinase mixtures at pH 2 led to a loss in ability of mixture to convert bovine plasminogen to plasmin. Plasminogen conversion activity could be re-established upon readdition of streptokinase. Results of repeated low pH treatments and readditions of streptokinase do not seem to support the hypothesis of the existence of a specific proactivator for formation of a singular plasminogen activator. Studies on the influence of a plasmin inhibitor, added at various stages of the interactions, on formation of plasminogen converting activity yielded results in agreement with the hypothesis that plasmin reacts, probably proportionally, with streptokinase to form a plasmin-streptokinase complex which has the ability to convert bovine plasminogen to plasmin.

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## Serum Lipoproteins and Glycoproteins in Aminopterin Treated Rats.* (25389)

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We observed(1) alterations in serum protein patterns of aminopterin treated folic acid deficient rats. Folic acid is involved in lipid metabolism(2-4). Lipids and the bulk of carbohydrates in blood are transported in association with proteins. The nature and composition of these lipoproteins and glycoproteins might reflect basic changes in metabolism and thereby give information on the nature of these changes. In the present investigation different electrophoretic components of serum glycoproteins and lipoproteins were determined in aminopterin-treated folic acid deficient and pair-fed normal rats. Total lipid, free cholesterol, ester cholesterol, phospholipid and neutral fat were also estimated in the serum of these animals.

*Methods.* Selection of rats, production of folic acid deficiency by intraperitoneal injection of aminopterin and collection of blood samples from carotid artery were the same as described previously(1). Only clear serum was used. Total glycoproteins in serum were determined by the method of Weimer and Moshin(5) as modified by Winzler(6). Cholesterol was estimated by the method of Sobel and Meyer(7). Phospholipid was estimated by determination of P of the serum extract(8) and multiplying the value by 25(9). Total serum lipid was determined by the method of Bragdon(10). Neutral fat was calculated by subtracting the figure for total cholesterol and phospholipid from the value for total lipid. Ionographic separation of lipoprotein and glycoprotein fractions of sera were carried out by the paper electrophoretic technic described earlier(1,11). For glycoproteins .01 cc and .04 cc serum were applied on different parallel paper strips and electrophoresis continued for 16 hours with a current of 4 ma and 200 volts in a barbiturate-barbituric acid buffer of pH 8.6 and ionic strength .05. The strip with .01 cc serum was stained with bromophenol blue for proteins, and used as reference strip in subsequent measurement of polysaccharides. Strips with .04 cc serum were stained for glycoproteins by the method of Koiw and Gronwall(12), modified by Roboz *et al.*(13), and based on histochemical technic described by Hotchkiss(14). Polysaccharides were indicated by violet bands. Densitometric evaluation of strips was performed immediately after staining, as the color was not permanent and the unstained portion of the paper turned pink after exposure for several hours in air. The densitometric curves of the different glycoprotein bands were measured by planimeter. For lipoproteins, the prestaining procedure of McDonald and Bermes(15) was followed. .05 cc of stained serum for lipoprotein and .005 cc of serum for protein determination were applied to parallel adjacent strips and electro-

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TABLE I. Serum Lipoproteins and Glycoproteins in Rats (mg/100 cc).

| | Normal (5) | Aminopterin treated (5) | "t" |
|---|---|---|---|
| $\alpha$-lipoprotein | 82 ± 22* | 85 ± 18 | .11 |
| $\beta$- " | 153 ± 23 | 319 ± 21 | 5.35 |
| "O" fraction | 227 ± 18 | 444 ± 31 | 6.03 |
| Free cholesterol | 12 ± 2 | 28 ± 2 | 6.40 |
| Ester " | 26 ± 3 | 40 ± 1 | 4.8 |
| Phospholipid | 183 ± 26 | 274 ± 76 | 1.1 |
| Neutral fat | 241 ± 45 | 506 ± 72 | 3.1 |
| Total lipid | 462 ± 30 | 848 ± 59 | 5.8 |
| $\alpha_1$-glycoprotein | 98 ± 8 | 105 ± 8 | .61 |
| $\alpha_2$- " | 28 ± 2 | 27 ± 4 | .25 |
| $\beta$- " | 47 ± 7 | 43 ± 3 | .55 |

* Mean ± S.E. Figures in parentheses indicate No. of animals.

phoretic separation continued for 5 hours at 4 ma and 200 v. in a barbiturate-acetate buffer of pH 8.6. A better resolution with barbiturate-acetate buffer than barbiturate-barbituric acid buffer was observed. The lipoprotein zones appeared blue against a white background. The ionograms were scanned immediately after drying since the patterns were observed to fade with time, and areas of the different zones were measured. Swahn(16) had shown that uptake of Sudan Black B by whole serum on filter paper was correlated with total lipid content of serum. Total area of the plotted curve was, therefore, equivalent to total serum lipid content of sample used for electrophoresis as determined by the chemical method. Glycoprotein and lipoprotein concentrations of different fractions were calculated from total polysaccharides and lipid contents respectively. The significance of deviations from normal mean values was computed by the "*t*" test.

*Results* are given in Table I. Aminopterin treated rats showed significant increase in total lipid, free cholesterol, esterified cholesterol and neutral fat. Phospholipid values did not change. There was no change in the $\alpha$-fraction but an increase in the $\beta$ and O-fraction of serum lipoproteins. Glycoproteins showed 3 components migrating with the mobility of $\alpha_1$, $\alpha_2$ and $\beta$ globulins as observed in man, dog and rabbit(17). There was no significant change in distribution of different glycoprotein fractions and in total glycoproteins in aminopterin treated rats.

*Discussion.* Hyperlipemia and hypercholesterolemia observed in the aminopterin treated folic acid deficient rats might be due to diminution of serum albumin(1,18) or to the lipotropic effect of folic acid(2,3). It appears that in folic acid deficiency there is a shift in distribution of fatty acids with a relative increase in neutral fat, free and esterified cholesterol. Folic acid accelerates methyl group neogenesis(19-22) and its deficiency is likely to affect the supply of choline and other methyl donors leading to changes in blood serum lipids. Lipids found in the "O" band are associated only to a small extent with $\gamma$-globulin. They consist mainly of substances which have been adsorbed on filter paper such as chylomicrons containing neutral fat and $\beta$-lipoproteins(23). The observed increase in O-fraction is possibly due to increased level of neutral fat which is also confirmed by chemical estimation. Serum cholesterol is mainly present in the $\beta$-lipoprotein(24). Increased serum cholesterol of aminopterin treated rats is possibly responsible for the increased $\beta$-lipoproteins as observed by paper electrophoresis. In contrast to lipids, distribution of protein bound carbohydrates did not change in aminopterin-treated rats. The magnitude of changes in distribution of serum lipids indicated more severe disturbances of lipid metabolism than their metabolism of carbohydrate in aminopterin-treated rats.

*Summary.* Different fractions of serum lipoproteins and glycoproteins were determined by paper electrophoresis in aminopterin-treated folic acid deficient and paired fed normal rats. Total lipid, free cholesterol, ester cholesterol and neutral fat were also estimated in serum of these animals. In aminopterin-treated animals there was significant increase in $\beta$-lipoprotein and O-fraction, no change in the $\alpha$-lipoprotein and increase in total lipid, neutral fat and free and ester cholesterol. There was no change in glycoprotein fractions of serum. The lipid metabolism is much more disturbed than carbohydrate metabolism in folic acid deficiency.

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## Serum Amylase Changes Due to Antipancreas Serum. (25390)

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Revival of interest in effects of antiorgan serum on normal animals has been directed largely toward the heart, kidney and thyroid. An additional endocrine organ, the pancreas, has been the subject of 2 conflicting reports. In one study murine pancreas demonstrated no serologic activity(1), in the other(2) it was shown that rat pancreas stimulates production of precipitating antisera in rabbits. The latter report suggested structural peculiarities of rat pancreas were in part responsible for its poor antigenicity, and the poor concentration of radiolabeled antibody when administered passively. The work presented here shows the discrete guinea pig pancreas is antigenic as determined by *in vitro* and *in vivo* serologic reactions and that its antiserum produces pancreas pathology and alters serum amylase levels.

*Materials and methods.* After removal of the pancreas from several ether-anesthetized guinea pigs, a 10% w/v homogenate was prepared in cold saline in Waring blendor. Immunization of male rabbits was performed according to Estes(3) with the complete homogenate. When the precipitating titer of undiluted immune serum reached 1:500 (supernatant of 10% suspension diluted 1:50), the rabbits were bled and serum collected. In view of ultimate use of antisera, a biologic assay seemed desirable and the passive cutaneous anaphylactic (PCA) method of Ovary (4) was chosen. Four hours after dermal sensitization with 0.1 ml of different antisera, 1 ml of a 50-50 mixture of 1% Evans blue and supernatant from the 10% pancreas extract was given intracardially. Reactive sites were measured after 45 minutes. To determine if injections of antiserum into normal guinea pigs would induce pathologic or enzyme changes, 2 intraperitoneal injections of 5 ml of pooled antiserum were given, one the same day and one the 4th day after base level serum amylase determinations were completed(5). On 5th day, serum amylase was again determined, the animals sacrificed, and the pan-

TABLE I. Serologic Reactions of Guinea Pig Pancreas with Its Antiserum.

| Rabbit | Precipitin titer dilution of antigen | PCA, mm × mm |
|---|---|---|
| APS-2 | 1:500 | 17.5 × 21.5 |
| -3 | 1:1000 | 24 × 32 |
| -4 | ” | not tested |
| -5 | ” | 20 × 22 |
| -6 | ” | 15 × 17.5 |
| NRS-1 | <1:10 | 1 × 1.5 |
| -2 | ” | 1 × 2 |
| -3 | ” | 1 × 1 |

APS—Immunized with pancreas antigen.
NRS—Normal rabbit serum.
PCA figures are avg of 2 animals.

TABLE II. Effects of Antipancreatic Serum on Normal Recipient Guinea Pigs.

| | Interstitial pancreatitis | Pancreatic serositis | Serum amylase changes | |
|---|---|---|---|---|
| | | | Increase | Range |
| NRS | 1/7* | 2/7 | 384 units | –600 to +1600 |
| APS | 4/7 | 7/7 | 931 ” † | –250 to +2900 |

* No. positive/No. tested. † 9 animals.

creas removed for histopathologic examination. For precipitin, PCA, and passive immunizations parallel experiments with normal rabbit serum were used as controls.

*Results.* Serologic reactions of the antipancreas (APS) and normal rabbit sera (NRS) are recorded in Table I. There seems to be no question that an antibody does exist since both tests are positive in every instance. Pre-immunization titers of experimental rabbits were less than 1:10 or the same as the normals listed. The antipancreas sera were also tested for guinea pig erythrocyte agglutinins and lysins, as well as serum and heart extract precipitins, all of which were negative. The positive PCA reactions were a deep blue in color with a small central ring characteristic of intense reactions.

Examination of hematoxylin, eosin stained sections of pancreas from the APS and NRS treated animals revealed the former to have definite pancreatic serositis in every instance and an acute interstitial pancreatitis in 4 of 7 animals examined (Table II). Although one animal of the NRS group is stated as having pancreatitis, it was not remarkable; serositis was present in 2 controls. Post-injection serum amylase increased in both groups; however, the APS treated animals increased 2.4 fold the NRS group. These figures include 2 animals in the APS treated group that were not examined histologically. An additional 4 animals given antiheart serum revealed an average increase of 366 units of serum amylase, essentially the figure for normal controls.

*Discussion.* Anti-endocrine organ sera seem to have either of 2 biologic effects depending upon antigenicity of the endocrine hormones. If the hormones are antigenic, the antiserum will contain antibodies which neutralize the hormone on passive immunization of normal animals. This seems to be the situation with antipituitary and antithyroid sera which depress growth rates(6) and $O_2$ consumption(7) respectively. Other endocrine antisera lacking antihormones cause tissue damage and excessive release of the hormone with typical changes in physiology of the animal(2).

In our instance the pancreas was damaged sufficiently to cause release of amylase which was not neutralized by any anti-amylase component of the serum sufficiently to depress blood levels and so simulates the second condition above. This increase in a serum enzyme due to immunologic tissue damage by a specific anti-organ serum is a finding which may be extendible to other organs known to be the source of specific enzymes.

*Summary.* As determined by an *in vitro* and an *in vivo* test, guinea pig pancreas has been found to be antigenic. Its antiserum produced pancreas pathology and serum amylase increases when administered passively to normal guinea pigs.

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# Release of Inorganic Iodine from Thyroid Gland in Initial Phase of TSH Action. (25391)

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It has been well established that TSH accelerates release of thyroid hormone from the thyroid gland and increases uptake of inorganic iodine by the thyroid gland(1,2,3,4). While the acceleration of the hormone release takes place within several minutes after injection of TSH, there is a latent period of several hours before the thyroid gland starts to increase its uptake of inorganic iodine(1,5,6,8) and few studies have been reported on the behavior of the thyroid gland in handling inorganic iodine shortly after injection of TSH. Our observation that TSH releases the inorganic iodine from the thyroid in the early phase of its effect prompted this report.

*Materials and methods.* Seventeen male dogs weighing about 10 kg and maintained on a low iodine diet for 2 weeks were utilized. In 13 dogs, 3 to 7 days before the experiments, 0.5 to 2 mc of carrier free $I^{131}$ were injected intramuscularly, while in the other 4 dogs, a similar amount of $I^{131}$ was injected 20 hours before the experiments. Under morphine anesthesia (100 mg), a midline incision was made in the neck to dissect out the thyroid gland and its vessels. The thyroid venous blood samples were collected from polyethylene catheters inserted into the inferior thyroid veins. A catheter was placed in the femoral artery to collect arterial blood samples and to measure arterial blood pressure. During the experimental period, about 10,000 units of heparin in saline were infused intravenously. Blood transfusion was used if necessary, to maintain constant blood pressure.

*Method.* In 14 dogs, after control period of 30 minutes to 2 hours, one USP unit of TSH in 10 ml saline was injected intravenously, and the effect of TSH observed for 2 to 10 hours thereafter. In 2 dogs, observation was started 8 hours after administration of TSH to determine duration of its action. At intervals of 15 to 30 minutes during control and experimental periods, blood samples were collected from catheters in the thyroid veins and femoral artery, and blood pressure at each time was recorded. Blood flow of the thyroid vein was estimated by volume of blood collected during each interval. Measurement of $PBI^{131}$: Samples from thyroid veins and femoral artery were centrifuged to separate 1 ml of plasma. Radioactivity of the plasma, measured in well type scintillation counter for 5 minutes, represented total plasma radioactive iodine. Five ml of 20% trichloroacetic acid was added in the same tube to precipitate the protein bound iodine, and the precipitate was washed twice with 5 ml of 10% trichloroacetic acid. The activity of the precipitate, redissolved with 0.5 ml of 2 N sodium hydroxide, which was measured in a well type scintillation counter for 5 minutes, represented the $PBI^{131}$ concentration in 1 ml of plasma. Concentration of inorganic iodine was expressed by the difference between the activity of total and $PBI^{131}$. Radiopaperchromatographic technic: 3 to 5 ml of plasma was acidified to pH 2-3 with 5 N HCl, extracted 3 times with 0.1 N HCl saturated butanol after addition of carriers, neutralized with 4 N $NH_4OH$ and evaporated to dryness in a 50°C water bath. The residue was dissolved in 0.2 ml of butanol and placed on Toyo filter

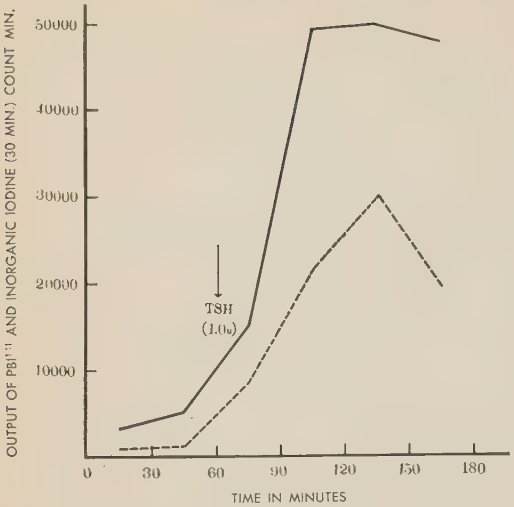


FIG. 1. Output of $PBI^{131}$ and inorganic iodine in dog administered $I^{131}$ 4 days before experiment. ——— PBI, - - - - - - inorganic iodine.

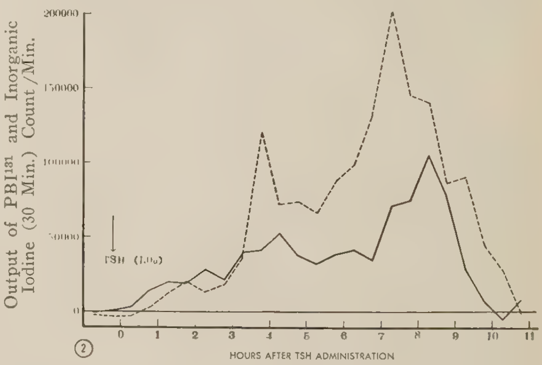
paper No. 50 in tertiary amyl alcohol-2 N ammonia or butanol acetic acid ascending system for 16 to 36 hours. The paperchromatogram was then cut into 0.5 cm horizontal strips for radioactivity measurement in a well type scintillation counter. Estimation of the output of $PBI^{131}$ and inorganic iodine from the thyroid was made by the following formula: output of $PBI^{131}$ (inorganic iodine) = (concentration of $PBI^{131}$ (inorganic iodine) in thyroid venous plasma – concentration of $PBI^{131}$ (inorganic iodine) in the femoral arterial plasma) × plasma flow of thyroid vein.

*Results.* Following a latent period of 15 minutes after one USP unit of TSH infusion, both $PBI^{131}$ and inorganic iodine concentration of thyroid venous blood rose sharply, and the increased concentration continued for about 10 hours, while concentration of femoral arterial blood remained at control level. Fig. 1 shows a representative experiment in which $I^{131}$ was injected 3 to 7 days previously, and Fig. 2 shows results in one experiment when $I^{131}$ was injected 20 hours before the experiment. In the second experiment (Fig. 2) the thyroid gland was accumulating inorganic iodine before injection of TSH and started to release iodide shortly after injection.

Fig. 3 depicts one of 2 dogs in which observation was started 8 hours after injection of TSH to determine duration of the release of inorganic iodine by TSH administration. About 10 hours after injection of TSH, release of inorganic iodine terminated and the thyroid gland started to accumulate iodide. A similar result was obtained in one other dog. In one experiment release of inorganic iodine was observed even when the dose of TSH was 50 USP milliunits. No change of blood pressure or thyroid venous blood flow was observed after TSH infusion.

The radiopaperchromatogram of the thyroid venous blood and femoral arterial blood before and after TSH revealed thyroxine, triiodothyronine, and inorganic iodine, but spots of iodotyrosine were not recognized. The ratio of the output of triiodothyronine to thyroxine showed a tendency to increase after TSH infusion.

*Discussion.* In our experiments, injection of 1 USP unit of TSH was followed shortly by release of both protein bound and inorganic iodine from the thyroid gland. The possibility that the increased output of inorganic iodine



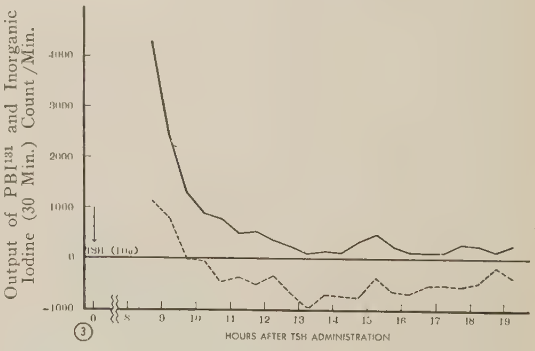


FIG. 2. Output of $PBI^{131}$ and inorganic iodine in dog administered $I^{131}$ 20 hr before experiment. ——— PBI, - - - - - - inorganic iodine.

FIG. 3. Output of $PBI^{131}$ and inorganic iodine in dog administered $I^{131}$ 3 days before experiment. ——— PBI, - - - - - - inorganic iodine.

was due to destruction of protein bound iodine before protein precipitation was most unlikely as thyroxine derivatives are known to be stable in the blood and the procedure was performed within 30 minutes after collection of samples. In one experiment the blood was collected directly into trichloroacetic acid without altering the results. The possibility that the increase of radioactivity in the unprecipitated fraction was due to free thyroxine, triiodothyronine, or iodotyrosine was excluded since most of the radioactivity was located in the inorganic iodine zone on the radiopaperchromatogram of this unprecipitated fraction. The observation that 50 USP milliunits of TSH released both inorganic and protein bound iodine indicated that this release was not caused by unphysiological dose of TSH.

Whether the release of inorganic iodine is due to deiodination of some products by the increase in thyroglobulin proteolysis, or due to change of permeability of cell membrane is still to be determined.

This phenomenon may explain the slowing of iodide disappearance in the plasma which occurs shortly after TSH administration in man(9) and also the increased urinary output of iodine within 24 hours after injection of TSH in man(7,8).

*Summary.* Measurement of inorganic $I^{131}$ and $PBI^{131}$ in thyroid venous blood obtained by catheterization was performed in 17 dogs to study the early effect of TSH. Following a latent period of about 15 minutes, output of both inorganic $I^{131}$ and $PBI^{131}$ started to increase sharply. The increased output continued for about 10 hours, and thereafter the thyroid gland started to accumulate inorganic iodine. The inorganic $I^{131}$ fraction, represented in the trichloroacetic acid supernatant fraction, was confirmed by radiopaperchromatography. TSH apparently releases inorganic iodine, in addition to PBI, from the thyroid in the early phase of its action.

We express our thanks to Prof. Okinaka and the medical staff of our department for their advice. The well type scintillation counter was purchased by grant from Rockefeller Fn.

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# Potentiation of Lathyrogenic Effect of Beta Aminopropionitrile in Turkey Poults.* (25392)

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Cyanoacetic acid (CAA) has been isolated from urine of rats and rabbits as a final product of metabolism of beta-aminopropionitrile (BAPN)(1,2). It is assumed that this conversion of BAPN to CAA is possible under the influence of the enzyme monoamine oxidase (MO). It has been shown that 1-isonicotinyl-2-isopropyl hydrazine (IIH) or iproniazid inhibits monoamine oxidase activity of rat liver *in vitro*(3), and the mode of action of IIH on mitochondrial MO of mammalian liver has been investigated(4). The inhibitory ef-

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TABLE I. Relative Toxicity of Beta Aminopropionitrile (BAPN) and Cyanoacetic Acid (CAA) to Turkey Poults.

| Supplements to basal diet | Avg wt, 5 wk, g | Avg leg score* | Mortality, % | Hemorrhage, % of original group | CAA in blood serum, mg/100 ml |
|---|---|---|---|---|---|
| None | 704.2 | 5.0 | 20 | 0 | 0–1† |
| .026% BAPN fumarate | 669.2 | 4.0 | 10 | 10 | 6.54 |
| .053% " " | 639.1 | 3.0 | 40 | 40 | 11.9 |
| .024% CAA | 749.5 | 5.0 | 20 | 0 | 11.3 |
| .048% " | 739.5 | 5.0 | 0 | 0 | 12.1 |
| .12% " | 751.9 | 5.0 | 10 | 0 | 29.7 |
| .24% " | 755.5 | 5.0 | 10 | 0 | 27.9 |

* Leg scores (avg of surviving birds): 1. Both legs severely bent; joints swollen. 2. One leg slightly, one severely bent; joints swollen. 3. Both legs slightly bent; joints swollen. 4. One leg normal; other slightly affected. 5. Normal.

† *cf.* ref.(7).

fect was also observed *in vivo,* as the monoamine oxidase activity of liver homogenates, liver mitochondria and brain mitochondria was inhibited almost completely in rats and guinea pigs after subcutaneous injection of IIH(5). The inhibitory activity of isonicotinic acid hydrazide (INAH) against MO was not as strong as that of IIH(3,5). INAH even at $10^{-3}$ molar concentration *in vitro* had slight or no effect on rat liver(3) and no apparent inhibitory activity *in vivo*(5). It was our purpose (1) to identify CAA as a metabolite in turkeys and measure its toxicity, (2) to study the effect of INAH and IIH when fed with and without BAPN fumarate, and (3) to determine whether non-toxic levels of n-propylamine would influence the toxicity of BAPN.

*Methods.* In all experiments day-old Broad Breasted Bronze turkey poults were used. They were kept in electrically heated, wire-floored batteries throughout the experiment. Each group contained 10 birds. The basal ration was a practical starting mash of composition given by Barnett, *et al.*(6). Feed and water were given *ad lib.* throughout the experiment. The birds were weighed at end of each week and any abnormalities found were recorded. Dead birds were examined for internal hemorrhage. Criteria of toxicity were depression of growth, mortality, thoracic or abdominal hemorrhage and leg deformity. The method for estimation of CAA in blood serum was that described by Sievert *et al.*(7). In experiments with n-propylamine, this compound was fed at predetermined levels from hatching to termination of experiment, and BAPN was fed from 2 weeks of age to end of experiment.

*Results.* The effects of feeding BAPN fumarate† and CAA are shown in Table I. CAA even at 0.24% of diet did not depress growth, increase mortality, or cause leg deformity or internal hemorrhage. BAPN fumarate at levels of 0.026% and 0.053% produced the usual toxic symptoms.

At termination of experiment, blood was taken by heart puncture and the blood sera analyzed for CAA content. Results have been presented elsewhere(7), but are included in Table I for comparison. Feeding of CAA produced blood serum levels more than twice as high as those produced by feeding BAPN, but the birds having these high blood levels showed no toxic symptoms.

Table II shows that isonicotinic acid hydrazide (INAH) alone caused depression of growth rate, but no hemorrhage and no leg deformity except at highest level. BAPN fumarate caused the usual toxic symptoms. The toxicity of BAPN was increased by INAH as evidenced by incidence of hemorrhage and the leg score of 1 in groups fed lowest level of BAPN in presence of the hydrazide.

Table III shows that 1-isonicotinyl-2-isopropyl hydrazine (IIH) increased toxicity of BAPN fumarate in the same manner as did INAH.

N-propylamine alone did not produce toxic symptoms, but caused some depression of

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† BAPN fumarate (2 BAPN-1 fumaric acid) was generously supplied by Abbott Labs., and 1-isonicotinyl-2-isopropyl hydrazine phosphate by Hoffmann-LaRoche.

TABLE II. Potentiating Effect of Isonicotinic Acid Hydrazide (INAH) on Toxicity of BAPN in Turkey Poults.

| BAPN fumarate, % of diet | INAH, % of diet | Avg wt, 4 wk, g | Mortality, % | Hemorrhage, % of original group | Avg leg score* |
|---|---|---|---|---|---|
| .0 | .0 | 469.4 | 10 | 0 | 5.0 |
| .02 | .0 | 525.5 | 0 | 0 | 4.1 |
| .04 | .0 | 457.7 | 30 | 30 | 3.4 |
| .08 | .0 | 153.0 | 100 | 40 | 1.0 |
| .0 | .04 | 352.2 | 10 | 0 | 5.0 |
| .02 | .04 | 233.0 | 80 | 30 | 1.0 |
| .04 | .04 | | 100 | 10 | |
| .08 | .04 | | ” | 0 | |
| .0 | .08 | 300.0 | 10 | 0 | 5.0 |
| .02 | .08 | 174.7 | 80 | 20 | 1.0 |
| .04 | .08 | | 100 | 20 | |
| .08 | .08 | | ” | 0 | |
| .0 | .16 | 156.5 | 80 | 0 | 3.0 |
| .02 | .16 | 235.7 | 80 | 20 | 1.5 |
| .04 | .16 | | 100 | 0 | |
| .08 | .16 | | ” | 0 | |

* *cf.* Table I.

growth rate, (Table IV, Exp. 23). When fed with BAPN, it increased mortality and incidence of hemorrhage and leg deformity. With n-propylamine, some birds that died did not show massive abdominal hemorrhages, but did have massive hemorrhages in the leg musculature. Incidence of each type of hemorrhage is given in Table IV.

Conversion of BAPN into CAA presumably occurs *in vivo* under the active influence of enzyme MO. It is suggested that when inhibitors like INAH or IIH are fed along with BAPN this conversion is inhibited and higher effective concentrations of BAPN result *in vivo*. This would account for the greater toxicity observed.

Similar effects of MO inhibitors on metabolism of several physiologically active amines have recently been reported(8,9,10). Such observations emphasize the importance of careful attention to possibly dangerous side effects of MO inhibiting drugs.

N-propylamine was fed prior to BAPN to stimulate MO formation. It was anticipated that animals conditioned in this manner might show an increased resistance to BAPN toxicity. However, the experimental results were just the opposite. Although no evidence is available to explain this finding, it is possible that the high level of n-propylamine may act competitively to retard the oxidation of BAPN.

*Summary.* Cyanoacetic acid (CAA) produced no toxicity symptoms when fed to turkey poults in quantities which induced relatively high blood levels. Two known inhibitors of monoamine oxidase, isonicotinic acid hydrazide and 1-isonicotinyl-2-isopropyl hydrazine at 0.04-0.08% of the diets were also not toxic, but when fed at same levels in

TABLE III. Potentiating Effect of 1-Isonicotinyl-2-Isopropyl Hydrazine (IIH) on the Toxicity of BAPN in Turkey Poults.

| BAPN fumarate, % of diet | IIH phosphate, % of diet | Avg wt, 5 wk, g | Mortality, % | Hemorrhage, % of original group | Avg leg score* |
|---|---|---|---|---|---|
| .0 | .0 | 742.5 | 0 | 0 | 5.0 |
| .02 | .0 | 630.8 | 20 | 20 | 4.6 |
| .04 | .0 | 555.0 | 60 | 30 | 3.0 |
| .0 | .04 | 610.0 | 0 | 0 | 4.8 |
| .02 | .04 | 400.1 | 90 | 60 | 1.0 |
| .04 | .04 | | 100 | 40 | |
| .0 | .08 | 627.6 | 0 | 0 | 5.0 |
| .02 | .08 | | 100 | 60 | |
| .04 | .08 | | 100 | 20 | |

* *cf.* Table I.

TABLE IV. Potentiating Effect of n-Propylamine (n-PA) on the Toxicity of BAPN in Turkey Poults.

| Exp. | n-PA, % of diet | BAPN fumarate,* % of diet | Avg wt, g† | Mortality, % | Hemorrhage, % of original group | | Avg leg score§ |
|---|---|---|---|---|---|---|---|
| | | | | | A.R.‡ | L.M.H.‡ | |
| 21 | .0 | .0 | 495.2 | 0 | 0 | 0 | 5.0 |
| | .0 | .079 | 424.0 | 10 | 0 | 0 | 3.4 |
| | .0 | .12 | 413.0 | 40 | 10 | 0 | 2.3 |
| | .5 | .079 | 396.6 | 50 | 10 | 0 | 2.6 |
| | .5 | .12 | 360.8 | 40 | 20 | 20 | 2.3 |
| 23 | .0 | .0 | 742.5 | 0 | 0 | 0 | 5.0 |
| | .0 | .08 | 601.6 | 20 | 20 | 0 | 3.5 |
| | .5 | .08 | | 100 | 40 | 10 | |
| | .5 | .0 | 623.4 | 10 | 0 | 0 | 5.0 |
| | 1.0 | .08 | | 100 | 10 | 10 | |
| | 1.0 | .0 | 448.8 | 20 | 0 | 0 | 5.0 |

* BAPN fumarate additions to diet were not started until beginning of third week of experiment.

† Durations of experiments 21 and 23 were 4 and 5 wk, respectively.

‡ A.R. = aortic rupture; L.M.H. = massive hemorrhage in leg muscles.

§ *cf.* Table I.

combination with BAPN, the characteristic toxicity symptoms of the latter substance were greatly enhanced. This is interpreted as the result of a decreased *in vivo* conversion of BAPN to CAA. N-propylamine at 0.5-1.0% of the diet did not itself produce toxic symptoms, but it enhanced the toxicity of BAPN.

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